IN VITRO STUDIES ON A PUTATIVE MOLECULAR MECHANISM OF ACTION OF VALPROIC ACID IN TREATMENT OF BIPOLAR DISORDER

BY

JEANNETTE M. WATTERSON

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ABBREVIATIONS AND DEFINITIONS

BIM - bisindolylmaleimide, an inhibitor of PKC

CMP-PA - cytidine monophosphate-phosphatidic acid; also CDP-DAG

CNS - central nervous system

DAG - diacylglycerol, a stimulator of PKC

FDA - U.S. Food and Drug Administration

GABA - gamma-aminobutyric acid, an [inhibitory] amino acid neurotransmitter

GAP-43 - 43 kD growth-associated protein

HN33 - immortalized hippocampal cell line derived from fusion of primary mouse hippocampal and human neuroblastoma cells

IMPase - *myo*-inositol 1-monophosphatase

IP - inositol [mono]phosphate

LY333531 - a PKC inhibitor designed by Eli Lilly and Company

MARCKS - myristoylated alanine-rich C kinase substrate

MBP - myelin basic protein

NGF - nerve growth factor

PC12 - rat pheochromocytoma (adrenal tumor) cell line

PI - phosphoinositide

PKC - protein kinase C

PDBu - phorbol 12,13-dibutyrate

RPA - RNase protection assay

SDS-PAGE - sodium dodecyl sulfate - polyacrylamide gel electrophoresis

VPA - valproic acid or valproate

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IN VITRO STUDIES ON A PUTATIVE MOLECULAR MECHANISM OF ACTION OF VALPROIC ACID IN TREATMENT OF BIPOLAR DISORDER

By

Jeannette M. Watterson

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Major Department: Pharmacology and Therapeutics

Valproic acid (VPA; trade name Depakote) is a broad-spectrum anticonvulsant which has of late been utilized as a treatment for bipolar disorder and has proven efficacious in treating a variety of both acute manic and chronic bipolar states. Lithium is currently the only other FDA-approved drug treatment for this disorder, and although it has traditionally been the first-line treatment of choice, there is a recognized need for alternatives, due to the low patient compliance rate resulting from troublesome side effects, narrow therapeutic index, and less than optimal patient response.

Very little is known about how either drug exerts its mood-stabilizing properties, and it has been the goal of this project to understand better the actions of VPA from a mechanistic standpoint.

Initial experiments were conducted utilizing an immortalized hippocampal cell line, the HN33 cells. Results indicate that at clinically relevant target serum concentrations, chronic, but not acute, VPA reduces expression of the myristoylated alanine-rich C kinase substrate (MARCKS), concomitant with increased GAP-43 expression. These effects are associated with altered activity and expression of protein kinase C (PKC), an important

effector in the CNS, and the effect of VPA on MARCKS appears to be dependent, at least in part, upon the activation/down-regulation of this enzyme. Furthermore, the observed effects are accompanied by alterations in cellular morphology and proliferation rate, which implies that VPA plays a major role in modulating neuronal plasticity.

Additional studies utilizing PC12 cells showed similar VPA-induced differentiation, with no evidence for neuroprotection following nerve growth factor-induced differentiation of cells. These studies bring us closer to elucidating the basis for the unique therapeutic profile of VPA, which includes both antiepileptic and antimanic/mood-stabilizing clinical applications, as well as unfortunate teratogenic effects (such as spina bifida, anencephaly, meningomyelocele, and encephalocele), and may provide further insight into the pathophysiology underlying bipolar disorder.

CHAPTER 1
INTRODUCTION

VPA is a broad-spectrum anticonvulsant shown in the last two decades to be efficacious in the treatment of bipolar disorder (manic-depressive illness). These two actions can be distinguished from each other by their time-courses, with anticonvulsant activity being observed much more quickly, on a scale of hours to days, in contrast to the days to weeks treatment required for mood-stabilizing activity. The precise mechanisms by which VPA exerts either its antiepileptic or antimanic effects are still unclear. VPA has been found to affect Na^+ flux, intracellular Ca^{++}, and brain gamma hydroxybutyric acid (GABA) levels; these actions probably relate to the anticonvulsant efficacy of this drug. The basis for its mood-stabilizing efficacy remains a mystery. In addition to its therapeutic actions, numerous studies have documented the deleterious effects of this drug on the developing nervous system, especially the embryonic neural tube. It is the goal of this project to elucidate further the mechanisms of action of VPA in brain, mainly through studies of its effects on intracellular signaling in one particular cell model, in an effort to understand better the underlying mechanism for this therapeutic profile. The specific aims of this project are to: (1) characterize the therapeutically relevant time- and concentration-dependent effects of VPA on PKC (activity and isozyme expression) and its substrates (MARCKS and GAP-43), using the hippocampally derived HN33 cells as a model system for neuronal effects of VPA; and (2) characterize the therapeutically relevant effects of VPA on the viability, growth, and morphology of both HN33 cells and PC12 cells. Based on preliminary evidence and previous reports, it is hypothesized that VPA will have a significant effect on the expression of PKC substrates MARCKS and GAP-43, that PKC

will be integrally involved in any such effects, and that cells will undergo an alteration in cell growth and morphology indicative of a transition to a more differentiated state.

VPA

VPA is a short-chain carboxylic acid that was first discovered to have antiepileptic effects in 1963, and has been utilized as an anticonvulsant in the United States for over 20 years (Meunier *et al.*, 1963; Ramsay, 1984). It first became available in the U.S. in 1978 as Depakene, the acid form of the drug. Five years later, the enteric-coated form, Depakote, was introduced (Penry and Dean, 1989). This particular formulation, the most popular form in use today, is a stable coordination compound containing equal proportions of valproic acid and the sodium valproate salt (McElroy *et al.*, 1989). VPA is striking in its structural difference from other known anticonvulsants, yet it is well known for its efficacy in treating a wide range of seizure disorders, giving it a broader therapeutic profile for epilepsy treatment than perhaps any other anticonvulsant yet studied. VPA is FDA-approved for treatment of simple and complex absence seizures, as well as mixed seizures which include the absence type. Additionally, VPA is effective in treating such primary generalized epilepsies as tonic-clonic, clonic, tonic, and myoclonic seizures, infantile spasms, and photosensitive epilepsy, as well as secondary generalized seizures and some partial seizures, including complex partial epilepsy (McElroy *et al.*, 1989).

A number of studies have investigated the putative mechanism(s) through which VPA exerts its anticonvulsant effects. No one signaling effect is likely to be responsible for the entire panorama of the anticonvulsant efficacy of this drug, and a number of molecular events appear to contribute to the net effect of reducing neuronal excitability. The ability of VPA to increase neurotransmission of the inhibitory amino acid GABA has been well documented. VPA may accomplish this through a variety of mechanisms - by increasing GABA levels through decreased degradation and increased synthesis and

release, or by increasing post-synaptic responsiveness to GABA through effects on the GABA-receptor complex or altered K^+ conductance, effectively dampening neuronal excitability (Chapman *et al.*, 1982; Rimmer and Richards, 1985; McElroy *et al.*, 1989; Penry and Dean, 1989; Joffe, 1993). Because anticonvulsant effects are often observed before total brain GABA levels become elevated, the VPA-induced increase in GABA levels alone may not account for the drug's anticonvulsant effect.

VPA, like other anticonvulsants, is known to block sustained high frequency repetitive firing of cultured neurons, due to its effect of slowing recovery of the sodium channel following inactivation (Albus and Williamson, 1998). At high concentrations, VPA has been shown to alter membrane potassium conductance, and relatively low concentrations of the drug hyperpolarize neurons, both effects suggestive of direct effects on neuronal membranes (Porter and Meldrum, 1995). Other studies have provided evidence for reduced excitatory neurotransmission by the amino acid aspartate (Chapman *et al.*, 1982). Still other reports have suggested that VPA works through inhibition of kindling, a model of epilepsy which holds that epileptic activity predisposes the system to increased frequency of seizures triggered by lower thresholds over time (Porter and Meldrum, 1995; McElroy *et al.*, 1989).

In summary, the various mechanisms proposed encompass three major net effects of VPA: (1) increased GABAergic synaptic inhibition post-synaptically; (2) reduction in the excitatory synaptic transmission ultimately responsible for epileptic kindling; and (3) reduction in repetitive firing via a direct effect on voltage-sensitive ion channels (Albus and Williamson, 1998). The broad spectrum of anticonvulsant activity of VPA is probably related to more than one of the molecular mechanisms demonstrated thus far. However, it is unclear whether such anticonvulsive effects may also account for the antimanic efficacy of VPA, and because little is known of the mood-stabilizing mechanism of VPA, much remains to be explained with regard to what kinds of intracellular changes might underlie this drug's mood-stabilizing effects.

Bipolar Disorder

In recent years, VPA has emerged as an effective alternative to lithium in the treatment of acute mania in patients suffering from bipolar disorder (Gerner & Stanton, 1992; McElroy *et al.*, 1992; Bowden *et al.*, 1994), and is also being used for long-term prophylactic management of this disorder. Bipolar disorder, also known as manic-depressive illness, is a debilitating psychiatric condition for which there is no known cause and only a limited range of treatment options available. It is estimated to affect about 1% of the population in this country (Bowden *et al.*, 1994). Patients afflicted with this affective disorder experience frequent, recurring episodes of extreme alterations in mood. Whereas the patient may appear normal between cycles, the episodes of mania and depression that characterize this disorder can leave the patient functionally incapacitated. The depressed phase involves motor activity alterations, impaired concentration, impaired social and occupational functioning, general helplessness, and may result in changes in sleep, appetite, weight, and energy level (Post, 1989). The manic episodes entail such disruptive symptoms as hyperactivity, explosive temper, impaired judgement, insomnia, disorganized behavior, hypersexuality, and grandiosity, any combination of which may lead to alienation from family and friends, indebtedness, job loss, and other major life problems (Bowden *et al.*, 1994). If untreated, this disorder has a tendency to worsen, with increasing severity, duration, and frequency of episodes over time (Post, 1989). There is a high rate of suicide in patients suffering from this disorder, estimated at about 15% in untreated cases (Bowden *et al.*, 1994).

Lithium has for over 40 years been the first-line drug therapy for both acute and prophylactic management of this disorder, but due to its narrow therapeutic index and many undesirable side effects (often resulting in compliance problems), the need for other viable treatment options has become a major concern (Post, 1989; Bowden, 1996). Although numerous treatments have been used experimentally in the past, including

antipsychotic drugs, calcium channel blockers (such as verapamil), high doses of thyroxine (T_4), serotonin precursors (such as L-tryptophan), and even electroconvulsive therapy (Joffe, 1993), results have not been consistently positive, and none is used extensively as a "mainstream" treatment at the present time. For example, the use of antidepressants is generally not considered because patients may be brought out of their depressive episodes only to be immediately submerged into the manic phase; further, certain drugs may increase the frequency of cycling of mood swings (Post, 1989). The anticonvulsants carbamazepine, lamotrigine, and gabapentin, as well as other pharmacological agents (clozapine, clonazepam, verapamil), have shown promise in treating bipolar disorder, but very few studies have been conducted and FDA approval has yet to be obtained for this clinical indication (Bowden, 1996; Joffe, 1993; Hollister, 1995). Because lithium is the approved first-line treatment, and has been used and studied extensively in the past, this project will be focused in part toward understanding the mechanism of action of bipolar drugs, based on what is known and has been reported experimentally for lithium.

VPA is emerging as a viable alternative or combinational drug treatment for bipolar disorder, and in fact, VPA may even have a wider spectrum of efficacy than lithium. Although VPA seems to be less effective in treating depression than in treating the manic phase of the disorder, there is accumulating evidence of its use in atypical (dysphoric/mixed) mania, rapid cycling, and secondary manias, which have poorer prognoses and are generally less responsive to lithium than the usual forms of the disorder (Calabrese & Delucchi, 1990; Calabrese *et al.*, 1992, 1993; Bowden *et al.*, 1994; Joffe, 1993; Brown, 1989). VPA has also been used to treat various conditions which are comorbid with bipolar disorder, such as migraines, panic disorder, bulimia, attention-deficit hyperactivity disorder (ADHD), and other brain-related illnesses, and VPA appears to be quite effective in treating lithium non-responders (Bowden, 1995; Joffe, 1993; Brown, 1989). The therapeutic index of VPA is considerably wider than that of lithium, thereby allowing for greater freedom in dosage adjustment, with less risk of toxicity.

Furthermore, the side effect profile of VPA is less intimidating than that of lithium. Whereas the side effects of lithium may be quite severe and disturbing to patients (thyroid, renal, and CNS effects, dermatologic and cardiovascular complications), VPA typically exhibits a different, more benign profile of side effects which are generally more acceptable to patients (gastrointestinal upset, weight gain, hair thinning, increased hepatic enzyme levels), and many of its bothersome side effects are alleviated over time or with a decrease in drug dosage (Bowden, 1995; Penry and Dean, 1989). The exception to this is the idiosyncratic, unpredictable and irreversible hepatic toxicity induced by VPA in predisposed patients on rare occasion. While this condition may be fatal if not detected in time, it affects only a very minimal portion of the population – the highest incidence reported was a rate of 0.01%. The majority of reported cases occur within the first three months of VPA therapy, and because signs common to hepatic failure are clearly present, patients can be monitored regularly for the first six months of therapy so as to reduce the risk of fatality and prevent any long-term damage. Despite this risk, the side effect profile of VPA is still considered to be safer and less bothersome than that of lithium.

Nonetheless, the mechanisms mediating the therapeutic properties of VPA in the treatment of bipolar disorder are not clearly understood. Whereas the therapeutic actions of VPA have been reportedly associated with potentiation of GABAergic transmission and other neurotransmitter effects (Chapman *et al.*, 1982; Post *et al.*, 1992; Loscher, 1993; Petty, 1995), these effects generally occur at rather high concentrations and may relate more to the anticonvulsant properties of VPA than to its mood stabilizing effects; see Figure 1.1 (Waldmeier, 1987; Motohashi, 1990). Further, as is the case for the time course of lithium, there is a delay of several days in the onset of clinical antimanic action of VPA, although loading strategies have demonstrated antimanic effects at times as early as 3 d (McElroy *et al.*, 1996). Thus, any mechanism postulated for the therapeutic action of these drugs must take this delayed onset into account (Manji *et al.*, 1995). Because VPA and lithium are currently the only two drugs approved by the FDA for treatment of

acute mania, comparisons of the actions of these agents and efforts to elucidate the mechanism(s) underlying the mood-stabilizing activity of both drugs is important to understanding the pathophysiology of this illness. Further, this approach may lead to the identification of new or existing drugs which may be useful in this application.

Mechanism of Action of Lithium in Treatment of Bipolar Disorder

Accumulating evidence from our laboratory and others strongly implicates receptor-mediated PI signaling in the mechanism of action of lithium in the brain (Berridge *et al.*, 1982; Godfrey, 1989; Lenox and Watson, 1994). Numerous studies have established lithium as an uncompetitive inhibitor of the enzyme inositol monophosphatase (IMPase), and the resulting accumulation of intermediates in the pathway which are responsible for additional downstream effects, including intracellular Ca^{++} release and PKC activation (see Figure 1.2; Post *et al.*, 1992). It was first suggested by Berridge and colleagues that depletion of free inositol, resulting from the accumulation of intermediates in the PI signaling pathway, is a major consequence of long-term lithium exposure (Berridge *et al.*, 1982; Berridge, 1989). This phenomenon would have greatest implication in certain areas of the brain which are relatively more inositol-limited by virtue of the blood-brain barrier as well as less capable of producing inositol *de novo*. Further, because the PI signaling cascade is linked to G protein-coupled muscarinic receptor (m_1, m_3, m_5) activation, areas of the brain undergoing the highest rate of receptor activation may predictably be preferentially affected by lithium. Indeed, studies in our laboratory have provided evidence for an inositol-driven attenuation of the effects of lithium in HN33 cells, as well as potentiation by a muscarinic receptor agonist (carbachol) and prevention/reversal by a muscarinic receptor antagonist (atropine) (Watson and Lenox, 1996). Following exposure of HN33 cells to therapeutic concentrations of lithium (1 mM), MARCKS protein expression is significantly down-regulated (Lenox *et al.*, 1996;

Watson and Lenox, 1996). This MARCKS reduction only occurs in the relative absence of inositol, for supplementation of inositol even at concentrations as low as 5 μM prevents the lithium-induced down-regulation of MARCKS (Watson and Lenox, 1996; Watson *et al.*, 1998; see also Table 3.2). Similarly, the concomitant exposure of HN33 cells to both lithium and the muscarinic agonist carbachol results in an increase in the level of MARCKS protein down-regulation observed, and exposure of cells to the muscarinic antagonist atropine reverses or prevents the lithium-induced reduction in MARCKS expression (Watson and Lenox, 1996; Watson *et al.*, 1998; see also Table 3.1). These findings have provided further support for the PI signaling hypothesis.

Studies of the molecular mechanism of action of lithium in brain have focused largely on its modification of PI signaling, particularly the lithium-induced accumulation of intermediates such as DAG and the resulting stimulation of PKC. Such a PI-mediated mechanism may account for the effects of lithium on PKC-regulated events, including protein down-regulation/degradation and transcriptional and translational events. In contrast, little evidence has been found to support a similar PI mechanism for VPA. Vadnal and Parthasarathy (1995) showed that VPA had no effect, either stimulatory or inhibitory, on IMPase partially purified from bovine brain. Further, lithium and carbamazepine (a less commonly used antimanic agent) exhibited opposite effects on IMPase, suggesting that the enzyme is not a common site of action for these two mood-stabilizing medications. Dixon and Hokin (1997) reported that in cerebral cortical slices, VPA caused no accumulation of inositol monophosphates, inositol bisphosphates, or inositol 1,3,4-trisphosphate, all of which accumulated following lithium exposure. Based on these observations, the depletion of inositol is not universally applicable as the basis for the antimanic action of mood-stabilizing drugs.

Alternatively, it has become apparent that the action of chronic lithium in the brain, which follows as a result of its interaction with the PI signaling pathway, is mediated through subsequent regulation of PKC and the down-stream posttranslational modification

of select protein substrates (Lenox, 1987; Manji and Lenox, 1994; Watson and Lenox, 1996). Further, the clinical effects of lithium may be mediated, at least in part, through these actions. Although VPA apparently does not share the property of PI signaling inhibition (Vadnal and Parthasarathy, 1995; Dixon and Hokin, 1997), the question remains as to whether it affects PKC, as previous studies have suggested (Chen *et al.*, 1994; O'Brien and Regan, 1998). It is a goal of this project to address further the potential role of PKC in the action of VPA in the brain, especially as such effects might relate to the mood-stabilizing properties of this drug.

PKC and its Substrates MARCKS and GAP-43

PKC exists as one of at least 11 structurally-related isozymes in mammals, many of which are Ca^{++}-activated and/or DAG-dependent, and have been implicated in numerous cellular responses associated with regulation of signaling and long-term events including ion channel and gene regulation (Newton, 1995; Nishizuka, 1995). Numerous studies have suggested a role for PKC in the long-term action of lithium (Lenox, 1987; Manji and Lenox, 1994), and recent data have provided evidence that chronic VPA exposure alters PKC activity as well as the expression of α and ε isozymes in non-neuronal cells (Chen *et al.*, 1994; Manji *et al.*, 1996b). Previous studies have demonstrated that chronic (but not acute) lithium treatment of rats, resulting in clinically relevant brain concentrations, produces a significant reduction in the PKC substrate MARCKS in the hippocampus, which persists beyond treatment discontinuation (Lenox *et al.*, 1992; Manji *et al.*, 1996a). This lithium-induced down-regulation of MARCKS has also been demonstrated in an immortalized hippocampal cell line (Watson and Lenox, 1996), in which it was previously demonstrated that phorbol esters, which directly activate PKC, down-regulate MARCKS protein expression in a PKC-dependent manner (Watson *et al.*, 1994).

MARCKS is a prominent and preferential substrate in the brain for PKC, which by virtue of phosphorylation, regulates the cellular localization and activity of this protein. MARCKS binds calmodulin in a calcium-dependent fashion and cross-links filamentous actin, and has been implicated in cellular processes associated with cytoskeletal restructuring and neuroplasticity, e.g., transmembrane signaling and neurotransmitter release (Aderem, 1992; Blackshear, 1993). In fact, previous studies in mutant mice lacking the MARCKS gene have shown that MARCKS expression is essential to normal CNS development in animals (Stumpo *et al.*, 1995). Insofar as MARCKS may also represent a molecular target for mood stabilizers in the brain (Lenox and Watson, 1994; Watson and Lenox, 1996), it should be of benefit to examine its potential regulation by VPA as well as by other psychotropic agents.

The growth-associated protein GAP-43 is a second protein thought to be integral to normal neuronal development and differentiation, as it is expressed at high levels during nervous system development and is associated with neurite extension (Jap Tjoen San *et al.*, 1992; Aigner and Caroni, 1993; Strittmatter *et al.*, 1995). GAP-43 shares a number of properties with MARCKS, including ability to bind actin and calmodulin (albeit in a Ca^{++}-independent fashion), as well as regulation by PKC (reviewed in Benowitz and Routtenberg, 1997). Inasmuch as previous studies have provided evidence for an inverse regulation of MARCKS and GAP-43 in certain regions of rat brain (McNamara and Lenox, 1998), it may be instructive to assess the relative levels of GAP-43 observed concomitant with VPA-induced alterations in MARCKS expression. Investigation of these two related but differentially regulated proteins will likely provide insight into the molecular mechanism of this drug, as will examination of this drug's effects on PKC, the primary enzyme responsible for the direct regulation of both of these proteins.

Developmental Effects of VPA

In addition to the therapeutic efficacy of VPA, this drug exhibits its share of drawbacks, the most severe of which are its effects on the developing embryo. VPA has been known for years to be a developmental teratogen. In recent years, women who are pregnant or even of childbearing age are encouraged to supplement their diets with adequate amounts of folic acid, for supplementation with this nutrient and its metabolites has been associated with decreased frequency of neural tube defects (including spina bifida, anencephaly, and encephalocele) in offspring of VPA-treated and nontreated mothers (Smithells *et al.*, 1981; Rhoads and Mills, 1986; Trotz *et al.*, 1987; Nosel and Klein, 1992; Ehlers *et al.*, 1996). Numerous studies have investigated this phenomenon, yet it remains unknown how VPA causes these developmental defects or how folate supplements are able to prevent such effects. Previous work has demonstrated that VPA inhibits cell proliferation through arrest of the cell cycle at mid G1 phase (Martin and Regan, 1991), as well as induces differentiation of neuroblastoma and glioma cells (Regan, 1985), which would suggest that VPA is interfering with development through manipulation of the proliferation rate and differentiation state of cells central to nervous system structure. Such effects on cell growth and morphology should be considered in any putative model for the molecular mechanism of this drug, and this study will therefore investigate the hypothesis that VPA exerts effects on both cell proliferation and cell morphology which may account for its teratogenicity.

Summary

Experiments designed to elucidate the short- vs. long-term intracellular actions of VPA will facilitate the development of a model which may explain, in part, the unique therapeutic profile exhibited by this drug. Initial VPA studies have been modeled after

those conducted previously or in parallel for lithium, so that comparisons may be made which might shed light on the basis for the common mood-stabilizing therapeutic action of these two drugs. In summary, thus far, it has been shown that in the HN33 cell model, lithium elicits a down-regulation in MARCKS primarily through its effect on PI signaling, as evidenced by the carbachol-driven potentiation and either inositol- or atropine-driven attenuation of effect (Watson and Lenox, 1996; Watson *et al.*, 1998). This lithium-induced MARCKS reduction occurs over a chronic time course, requiring days to weeks, and the effect is greater in the soluble rather than membrane fraction of the cells (Lenox *et al.*, 1996; Watson and Lenox, 1996). A similar time course of MARCKS mRNA reduction is also observed (Watson and Lenox, 1997). Further, chronic lithium administration elicits a significant down-regulation of MARCKS protein *in vivo*, in a rat model (Lenox *et al.*, 1992). As for the effects of lithium on cell viability and related parameters, no alteration in cell growth rate or morphology has been observed following acute or chronic lithium exposure (Watson and Lenox, 1996), and no alteration in expression of another prominent PKC substrate, GAP-43, was observed (Watson and Lenox, 1997). Lithium exposure results in predictable changes in PKC activity (with initial activation followed by chronic down-regulation), as well as alterations in expression of the two PKC isoforms α and ε (Watson and Lenox, 1997), similar to findings previously reported by Manji *et al.* (1996a,b).

Applications of research into the mechanism of action of VPA will be multifold. In addition to providing a better understanding for the efficacy of VPA in treating bipolar disorder, a clearer picture of the pathophysiology underlying this illness, and identification of new or existing drug treatments, investigation of the molecular bases for these effects may foster a better understanding of how VPA exerts its deleterious developmental effects and the development of potential therapeutic interventions to prevent or correct such defects.

Specific Aims

Characterize the Therapeutically Relevant Effects of VPA on PKC and Its Substrates.

MARCKS and GAP-43 are known to be important in modulating central nervous system (CNS) plasticity and intracellular signaling, by virtue of the regulatory enzyme PKC. Using hippocampally derived HN33 cells, I will characterize both acute and chronic VPA-induced alterations of PKC expression and activity, as well as on levels of expression of MARCKS and GAP-43.

Effects of VPA on MARCKS protein expression. Previous studies showed that chronic lithium exposure at therapeutic concentrations resulted in down-regulation of MARCKS protein expression in HN33 cells, and involved alterations in PI signaling. After (1) establishing the concentration-response relationship for the effects of VPA on MARCKS protein, I will (2) establish the time course of action of VPA at therapeutic concentration, as well as (3) the time course of system recovery after VPA withdrawal. In addition, I will assess the importance of (4) inositol concentration and (5) muscarinic receptor/Gq activation, in VPA-induced MARCKS effects. Furthermore, I will (6) investigate the effects of combined VPA and lithium treatment. Effects will be examined in both cytosolic and membrane fractions of cells. It is predicted that VPA will have effects similar to those of lithium, i.e. significant down-regulation of MARCKS protein expression following chronic (but not acute) exposure to concentrations of the drug within a therapeutically relevant range, and that MARCKS will return to basal levels after drug removal. In contrast to lithium, there is no evidence for VPA-induced modulation of the

PI signaling pathway, so no effects by inositol or carbachol (muscarinic receptor agonist) are expected.

Effects of VPA on MARCKS mRNA. Preliminary studies have provided evidence for a lithium-induced reduction in MARCKS mRNA. I will establish the time course of therapeutically relevant VPA-induced effects on MARCKS mRNA levels. It is predicted that VPA will elicit down-regulation of MARCKS mRNA is a fashion similar to that of lithium, but following the time course of VPA-induced alterations in MARCKS protein.

Effects of VPA on GAP-43 expression. I will establish a dose-response and time course for therapeutically relevant VPA-induced effects on GAP-43 protein levels in both cytosolic and membrane cellular fractions. Although there are no previous reports for VPA- or lithium-induced alterations in GAP-43 expression, such effects are a likelihood given preliminary evidence for VPA-induced morphological alterations, and may partially account for the plastic changes (such as synaptic remodeling) essential during therapy for seizure and mood disorders.

Effects of VPA on PKC isozyme expression. I will establish the time course, at therapeutic concentration, of VPA-induced alterations in four PKC isozymes expressed in HN33 cells. Based on previous findings with lithium and VPA, which provide preliminary evidence for effects of each on PKC expression and activity, I predict that VPA will have significant effects on both PKC activity and isozyme expression, especially if alterations in MARCKS and GAP-43 are observed following chronic VPA exposure.

Effects of VPA on PKC activity. I will investigate the effects of therapeutic concentrations of VPA on PKC activity (as measured by phosphorylation of a PKC-specific substrate) over a broad time course from acute to chronic time points. VPA-

induced alterations in PKC activity are expected for the same reasons as described above in D.

Role of PKC in mechanism of action of VPA. Through the use of PKC inhibitors and antisense, which block PKC activity, I plan to further characterize A-E above; specifically, MARCKS and GAP-43 alterations, as well as morphological effects (see below), will be investigated following PKC inhibition via enzyme inhibitors and isozyme-specific antisense, in order to further define the role of PKC in these parameters.

Characterize the Physiologically Relevant Effects of Chronic VPA on Viability of HN33 Cells and PC12 Cells.

Effects of VPA on HN33 cell growth. I will investigate the effects of chronic VPA exposure at therapeutic concentrations on cell viability through observation of cell number and doubling time. The role of PKC in any observed effects will also be assessed. Based on limited previous studies in other cell lines, it is predicted that VPA will produce changes in cell growth which may help to explain, in part, the severe developmental effects associated with prenatal VPA exposure.

Effects of VPA on HN33 cell morphology. I will also investigate the effects of chronic, therapeutic VPA exposure on cell phenotype, looking specifically for differentiated morphology and other markers indicative of altered state of maturation. The role of PKC in any observed effects on cell differentiation will also be assessed. Despite the lack of evidence for a lithium-induced alteration in cell morphology, VPA-induced alterations in cell morphology are considered likely, given this drug's therapeutic profile (anticonvulsant and mood-stabilizing, yet teratogenic). Observed alterations in MARCKS

and GAP-43 will further support this hypothesis, given their integral role in CNS development and plasticity.

Effects of VPA on PC12 cell viability. Preliminary evidence for effects of VPA on cell growth and morphology leads me to investigate more in depth the effects of VPA on cell viability. Whereas the HN33 cell line is a sufficient model for the investigation of intracellular signaling phenomena as outlined in the studies above, post-mitotic cell lines may serve as better developmental models for investigation of such phenomena as neuroprotection or drug toxicity, and these cell lines have been better characterized for such investigations. Therefore, in order to investigate the potential of VPA to elicit either toxic or neuroprotective effects, NGF-differentiated and non-differentiated PC12 cells will be utilized. In light of the well-established neuronal toxicity of VPA exhibited during development, toxicity resulting from high levels of the drug would not be a surprise; however, preliminary studies in our laboratory have shown no evidence for toxic effects of this drug at therapeutic concentrations. Any cytoprotective effect of VPA has yet to be established, and there is currently no evidence in support of such an effect.

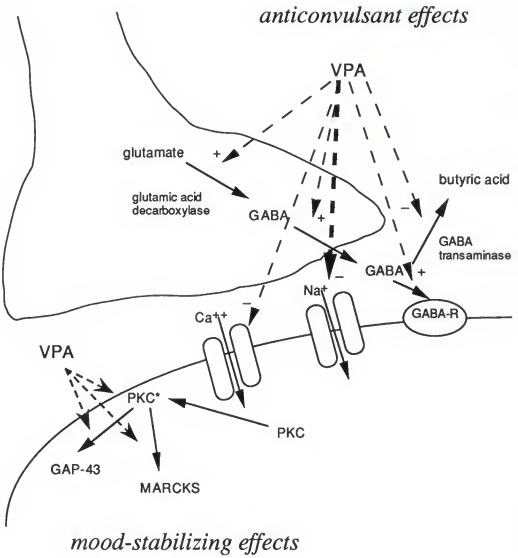
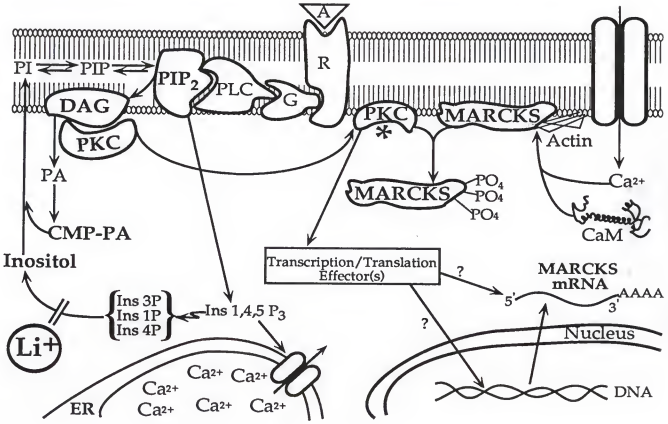


Figure 1.1. Molecular Effects of VPA in Brain. Depicted are (1) at right, the effects of VPA as previously established by other groups, and thought to be responsible for the anticonvulsant efficacy of the drug, and (2) at left, the long-term effects hypothesized to contribute to the mood-stabilizing efficacy of VPA. Of the short-term effects at right, increased GABA neurotransmission and effects on intracellular calcium are generally thought to contribute less than the effects of VPA on voltage-sensitive ion channels and the associated decrease in excitatory neurotransmission and neuronal firing. The hypothesized effects at left will be assessed in detail in the studies which follow.

Figure 1.2. Model for Lithium Regulation of MARCKS. (adapted from Lenox *et al.*, 1996). Following agonist occupancy of the receptor binding site, G-protein coupling mediates activation of the enzyme phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers diacylglycerol (DAG) and *myo*-inositol 1,4,5-triphosphate (Ins 1,4,5 P_3). DAG is an endogenous activator of PKC, and Ins 1,4,5 P_3 induces release of calcium from intracellular stores such as the endoplasmic reticulum. Lithium inhibits uncompetitively the enzyme inositol monophosphatase (IMPase), thereby inhibiting the recycling of inositol. This results in accumulation of metabolites in the DAG pathway during stimulation of receptor signaling. Activation of PKC* through both increased calcium and DAG initiates posttranslational events such as the translocation of PKC to the membrane where it phosphorylates the MARCKS protein, which then translocates to the cytosolic fraction. In the phosphorylated form, MARCKS no longer binds calmodulin or cross-links actin, thereby altering compartmental intracellular calcium signaling and affecting cytoskeletal restructuring. In addition, chronic lithium and receptor activation down-regulate MARCKS protein expression, which may result from down-stream transcriptional/translational events.


A
R
PI
PIP
PIP2
PLC
G
DAG
PKC
PA
CMP-PA
Inositol
Li+
Ins 3P
Ins 1P
Ins 4P
Ins 1,4,5 P3
ER
Ca2+
PKC
MARCKS
Actin
Ca2+
CaM
MARCKS
PO4
PO4
PO4
Transcription/Translation
Effector(s)
?
MARCKS
mRNA
5'
AAAA
3'
?
Nucleus
DNA

CHAPTER 2
MATERIALS AND METHODS

Chemicals

VPA (2-propyl-pentanoic acid) and other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise noted. For some drug comparison studies, additional VPA and VPA metabolites were obtained from Abbott Laboratories (Abbott Park, IL), and fluoxetine and LY333531 (a PKC inhibitor) were provided by Eli Lilly and Company (Indianapolis, IN). Tissue culture reagents were purchased through Gibco (Gaithersburg, MD; horse serum, RPMI and DME media), Hyclone (Logan, UT; fetal bovine serum), and Sigma (trypsin and gentamicin). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA), and RPA reagents were purchased from Ambion (Austin, TX).

HN33 Cell Culture

The immortalized hippocampal cell line HN33.dw(9/21) was used to assess effects of VPA on all parameters, including MARCKS protein and mRNA, GAP-43, PKC activity and expression, etc. HN33 cells were kindly provided by Dr. Bruce Wainer (Emory University, Atlanta, GA). These are derived from the fusion of primary neurons from the hippocampus of postnatal d 21 mice with the N18TG2 human neuroblastoma cell line, and exhibit morphological, immunological, and electrophysiological parameters characteristic of hippocampal neurons in culture (Lee *et al.*, 1990). These properties are described in Table 2.1. HN33 cells have been used in our laboratory as a model system to address the

mechanism of action of lithium, and we previously established the presence of a functional second messenger (PI) signaling pathway in these cells (Watson and Lenox, 1997). Furthermore, HN33 cells have recently been assessed and found to express at least two of the neurofilament triplet proteins (Watterson, unpublished data), which supports their consideration as phenotypically neuronal (Debus *et al.*, 1983; Shaw, 1991). Cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 5% defined fetal bovine serum (FBS) and 1 mg/ml gentamicin, with or without the addition of 0.6 mM VPA. The duration of exposures ranged from 0 to 14 d. VPA was added to cultures at time of plating, and culture medium was changed or cells were passaged every 3-4 d, with no replenishment of VPA between platings. Assays were performed when cells reached 80-90% confluence.

Table 2.1 lists the properties present (+) or absent (-) in HN33 cells and the two parent cell lines from which the HN33 were derived, primary mouse hippocampal neurons (21 d) and N18TG2 neuroblastoma cells. This table was adapted from that reported in Lee *et al.* (1990), in which the HN33 cell derivation and characteristics were first reported.

Table 2.1. Properties of HN33 Hybrid and Parental Cells

Tissue or Cell Line	Hybrid GPI Isozyme	Neuritic Processes	Excitable Membrane	NF-M	NGF Protein	NGF mRNA
Hippocampus	-	+	+	+	+	+
N18TG2	-	-	-	-	-	-
HN33	+	+	+	+	+	+

PC12 Cell Culture

PC12 cells obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 containing 10% heat-inactivated horse serum, 5% FBS, and 0.5 mM l-glutamine. Cultures were maintained at 37°C and 5% CO_2, and passaged every 3 d. To

assess VPA-induced differentiation, cells were exposed to 0.3 - 1.8 mM VPA for 2 d, and then neurite outgrowth was quantitated as described below. The cytoprotective assay was carried out following differentiation of cells by 7-d exposure to 100 ng/ml NGF, and subsequent NGF removal and replacement with 0.3, 0.6, and 1.2 mM VPA for 2 d. Viviana Puig is acknowledged for her extensive assistance in carrying out the technical aspects of these experiments utilizing the PC12 cells.

Neurite Quantitation

HN33 cells were cultured in the presence or absence of 0.6 mM VPA for 3-14 d, and process outgrowth was assessed using phase-contrast microscopy. A neurite was scored if its length was at least one respective cell body diameter. Treatments were compared for percentage of cells with one or more neurites and two or more neurites, and 150-900 cells were scored per treatment group. PC12 cells were similarly assessed, using the same criteria, with the aid of NIH Image 1.47 imaging software. Dan O'Donnell, Jeremy Grimes, Risha King, and Keith Templin are acknowledged for their participation in quantitation of neurite outgrowth in the PC12 cells.

Protein Quantitation by Western Blotting

Harvested cells were homogenized in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM PMSF, 2 mM DTE, and 10 μg/ml aprotinin. Cells were sonicated to disrupt cell membranes, and the soluble and pellet fractions were separated by centrifugation. The homogenate was centrifuged at 100,000 x g and the soluble fraction collected. The pellet was resuspended in buffer containing 0.1% Triton X-100 and solubilized for 30 min. Solubilized fractions were then centrifuged at 50,000 x g and the supernatant containing the solubilized membrane protein collected. Samples were

adjusted by addition or dilution to 0.05 % Triton X-100. Equal amounts of soluble and membrane cell protein (50 μg), as determined by the Bradford method, were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) in a Bio-Rad Trans-Blot electrophoresis apparatus at 100 V for 2 h using Towbin's buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% (v/v) methanol). All antibodies were diluted in TS buffer (20 mM Tris, pH 7.5, and 0.5 M NaCl); see Table 2.2 for dilutions and incubation times. Detection of the immune complex was performed using HRP-conjugated anti-rabbit or anti-mouse IgG, and the Pierce (Rockford, IL) enhanced chemiluminescence system. Western blots were quantified using NIH Image 1.47 software for densitometric analysis. Results are expressed as the percentage of protein present in drug-free controls grown in parallel and assayed on the same Western blot.

Ribonuclease (RNase) Protection Assay (RPA)

The RPA was performed with the RPAII kit using biotinylated antisense riboprobes (BIOTINscript and BrightStar system, Ambion). Biotinylated antisense riboprobes from the *Macs* (mouse gene coding for MARCKS) and the cyclophilin genes were generated and gel-purified. A fragment of *Macs* was cloned by RT-PCR into a TA cloning vector (InVitrogen, Carlsbad, CA) and restricted at a unique XmaI site. *In vitro* transcription using SP6 RNA polymerase and this construct as the template yields a 466 nt riboprobe and protects a 386 bp fragment of MARCKS mRNA. The cyclophilin probe (Ambion) protects a 103 bp fragment. For each sample, 15 μg of RNA was incubated with 1 ng of each probe at 44°C overnight. Non-hybridized RNA was digested with a mixture of RNases. Protected fragments were separated on a denaturing 5% PAGE and then transferred onto a nylon membrane to allow detection. Resulting autoradiographs were then quantified and the amount of *Macs* expression was normalized to the cyclophilin

internal control. Sharlynn Sweeney is acknowledged for her expertise in development of the probes, as well as her technical skill in carrying out the RPAs and quantitating results.

PKC Inhibitors

HN33 cells were exposed to the PKC inhibitor LY333531 at 1.0 μM (Eli Lilly and Company) concomitantly with 1 mM VPA for 24 h. Alterations in VPA-induced MARCKS down-regulation and GAP-43 up-regulation, as well as cell density and morphology, were measured and compared between control groups and LY333531-treated groups, each in the presence or absence of VPA. PC12 cells were exposed to the PKC inhibitor bisindolylmaleimide (BIM; Gibco, Gaithersburg, MD) at 200 nM for 48 h, and alterations in cell density and morphology were quantitated and compared between treatment groups.

PKC Activity

PKC activity was measured by the phosphorylation of myelin basic protein (MBP) 4-14 peptide (Upstate Biotechnology, Lake Placid, NY) and MARCKS peptide. Soluble and membrane cell fractions were prepared as described above. Ten microliters of the cell protein (1 μg/μl) were added to 100 μl of a reaction mixture containing 100 μM MBP, 100 μM $CaCl_2$, 20 mM HEPES (pH 7.5), 0.03% Triton X100, 10 mM $MgCl_2$, 100 μM ATP, and 0.0228 μCi/μl [γ-^{32}P]ATP, with or without 100 μg/ml phosphatidylserine and 20 μg/ml PMA. The mixture was then incubated at 30° C for 2 min. The reaction was stopped by the addition of 50 μl of ice-cold 450 mM phosphoric acid. Following a 5 min incubation on ice, 30 μl of each sample was spotted onto Whatman P81 filter paper. Filters were washed (x4) with a mixture of 150 mM H_3PO_4 and 10 mM sodium pyrophosphate, rinsed in ethanol and then acetone, then dried and radioactivity measured. Specific activity for

PKC was measured as nanomoles of ^{32}P/min/mg protein, and expressed as the difference between activity in the presence and absence of PMA/phosphatidylserine. Dr. David Watson is acknowledged for his contribution to the development of the PKC activity assay and his assistance in carrying out the experiments.

Statistical Analyses

Following consultation with a biostatistician, statistical differences between treatments were analyzed by one-way ANOVA, and post hoc comparisons were conducted using Fisher's PLSD test. In all cases, minimal acceptable level for significance was $p<0.05$. All results are reported as mean percentage of an untreated control group assayed in parallel, and error bars represent S.E.M.. Drs. Ronald Marks, Robert McNamara, and Mark Lewis are acknowledged for their assistance in identifying valid statistical methods for analyzing these data.

Table 2.2. Antibodies for Western Blots. This table lists the primary antibodies utilized in western immunoblots to detect each of the proteins of interest. For each antibody, the following information is reported: source from which antibody was obtained, dilution (in tris-saline, or TS, buffer) at which membrane was exposed to antibody, length of exposure of membrane to antibody, and secondary HRP-conjugated antibody used for amplification and detection of primary antibody, its source, dilution (in TS buffer + 0.2% Tween-20), and exposure time. The anti-MARCKS polyclonal primary antibody was raised against a glutathione-S-transferase MARCKS fusion protein designed in our laboratory, as detailed in Watson and Lenox (1996).

1° Ab	Source	Dilution	Incubation	2° Ab	Source	Dilution	Incubation
MARCKS	(custom made)	1:36,000	overnight	anti-rabbit	Bio-Rad	1:24,000	2 h
GAP-43	Boehringer-Mannheim	1:3,000	overnight	anti-mouse	Bio-Rad	1:20,000	2 h
PKC-α	Transduction Labs	1:1,000	2 h	anti-mouse	Bio-Rad	1:10,000	1 h
PKC-δ	Santa Cruz	1: 750	2 h	anti-rabbit	Bio-Rad	1:20,000	1 h
PKC-ε	Santa Cruz	1:1,000	2 h	anti-rabbit	Bio-Rad	1:20,000	1 h
PKC-ζ	Transduction Labs	1:1,000	2 h	anti-mouse	Bio-Rad	1:20,000	1 h

CHAPTER 3
EFFECTS OF VPA ON MARCKS AND GAP-43

Introduction

VPA has traditionally been used for its antiepileptic properties, which were first discovered in 1963 by Meunier *et al.*, and encompass a broad range of seizure disorders. Of late, VPA has been recognized as a suitable alternative to lithium, the traditional first-line drug, for both the acute and prophylactic treatment of bipolar disorder. VPA and lithium are currently the only two FDA-approved anti-manic drugs. Although lithium has been studied more extensively and will serve as the model upon which experiments in this study are based, the mechanism of anti-manic and mood-stabilizing action of this drug remains unclear, as does the etiology of the psychiatric disorder targeted by these drugs. Therefore, investigations into the mechanism(s) underlying the mood-stabilizing activity of both drugs is an important area of study.

MARCKS

Studies conducted in our laboratory, using both animal and cell models, have provided evidence for a significant down-regulation of MARCKS following chronic but not acute exposure to therapeutically relevant concentrations of lithium (Lenox *et al.*, 1992; Watson and Lenox, 1996). In immortalized hippocampal cells, Watson and Lenox (1996) first demonstrated that long-term (3-10 d) exposure to 1-10 mM LiCl resulted in a reversible, time- and concentration-dependent down-regulation of MARCKS protein.

Similarly, *in vivo* studies demonstrated that exposure of rats to chronic lithium (3-4 weeks), resulting in clinically relevant serum concentrations of 0.7-1.2 mEq/kg in brain, also reduced MARCKS in the hippocampus (Lenox *et al.*, 1992). Additionally, recent data from our laboratory suggest a time course of MARCKS mRNA regulation by lithium in HN33 cells similar to the time course of MARCKS protein down-regulation (with reductions evident by 3 d and persisting beyond 10 d), thereby indicating alterations in either transcription or post-transcriptional message stability (Watson and Lenox, 1997).

MARCKS expression is widely distributed, with highest levels normally expressed in brain, spleen, testis, and lung, and lower expression in heart, kidney, liver, and muscle tissues of mouse (Lobach *et al.*, 1993; Swierczynski *et al.*, 1996). In the brain, the greatest expression is in the most plastic regions, including the amygdala, striatum, olfactory bulb and cortex, and septum (Ouimet *et al.*, 1990; McNamara and Lenox, 1997), and MARCKS is localized to small dendritic branches and axon terminals of neurons and both cytoplasmic and membranous components of glia (Katz *et al.*, 1985; Patel and Kligman, 1987; Ouimet *et al.*, 1990; Rosen *et al.*, 1990). MARCKS cross-links actin and binds calmodulin in a Ca^{++}-dependent manner (these events are mutually exclusive; Sheu *et al.*, 1995; Allen and Aderem, 1995), and as a result, has been implicated in cell motility, secretion and trafficking, and cell cycle regulation (for reviews, see Aderem, 1992 and Blackshear, 1993). As is indicated by its name, the activity and expression of MARCKS are regulated in part through phosphorylation by PKC, though PKC-independent pathways may also play a role in its post-transcriptional regulation (Brooks *et al.*, 1992). The inhibition of PKC or mutation of the PKC phosphorylation sites of MARCKS results in defects in cellular motility (Allen and Aderem, 1995).

MARCKS is a protein for which the precise function is unclear but which is believed to be essential to normal CNS development, since mice lacking detectable mRNA and protein, due to gene (*Macs*) knockout, died perinatally, either before or within just hours after birth (Stumpo *et al.*, 1995). Developmental defects included defects in midline

events such as neurulation, fusion of the cerebral hemispheres, formation of the great forebrain commissures, and retinal and cortical lamination (Stumpo *et al.*, 1995). Ultrastructural examination revealed widespread neuronal ectopia, or abnormal neuronal pathfinding, during cerebrocortical development (Blackshear *et al.*, 1996). Whereas these findings do not preclude the possibility of more subtle ultrastructural or immunological defects in other non-neuronal tissues in which MARCKS is expressed, phenotypic alterations were not observed outside the CNS, and the data overwhelmingly support the notion that the mortality is primarily a result of the high frequency of midline defects in brain resulting from the MARCKS-deficient phenotype. Interestingly, this knockout phenotype was "rescued" by the expression of a supplemental human MARCKS gene, *MACS*, in that the human transgene contained all of the elements necessary for normal developmental expression of MARCKS, and was able to fully complement the MARCKS-deficient phenotype of the knockout mice (Swierczynski *et al.*, 1996).

The purpose of the following experiments is to investigate the effects of VPA on MARCKS expression, since significant effects were reported following chronic lithium exposure, as noted previously, and any common action of these two mood-stabilizing agents should be instructive as to their mechanisms of action. If MARCKS alterations are integral to the mood-stabilizing properties of these and other drugs, one would hypothesize that VPA should down-regulate MARCKS in a manner similar to that of lithium. Both concentration-response and time-course curves of VPA-induced effects will be established. These parameters address the therapeutic relevance of observed effects, since each drug exhibits its own clinical profile, including such characteristics as therapeutic serum concentration and latency to response. Previous studies have reported that the effects of lithium on MARCKS protein expression occur following chronic but not acute exposure to concentrations within the reported therapeutic serum concentration range of this drug, and that the observed down-regulation of MARCKS expression is reversible upon drug withdrawal. These findings are consistent with the known clinical

profile of lithium. Similar results are hypothesized following VPA exposure, since this drug exhibits similar clinical properties of delayed onset of antimanic drug action and delayed return to baseline following drug removal, over a therapeutic concentration range (50-125 μg/ml). The long-term effects of VPA will be the focus of these studies, for they may be important to the mood-stabilizing efficacy of this drug, whereas effects observed more acutely are generally credited to the anticonvulsant properties of VPA.

As mentioned in Chapter 1, accumulating evidence implicates receptor-mediated PI signaling in the mechanism of action of lithium in the brain (Berridge *et al.*, 1982; Godfrey, 1989; Lenox and Watson, 1994). Lithium is an uncompetitive inhibitor of the enzyme IMPase, and as such, it leads to accumulation of intermediates in the pathway responsible for additional downstream effects, including intracellular Ca^{++} release and DAG-mediated PKC activation (refer to Figure 1.2; Post *et al.*, 1992). It is hypothesized that depletion of free inositol, resulting from the accumulation of intermediates due to incomplete cycling of the PI signaling pathway, is a major consequence of long-term lithium exposure (Berridge *et al.*, 1982; Berridge, 1989). Such a phenomenon would have its greatest impact on certain areas of the brain which are relatively more inositol-limited, for example by virtue of the blood-brain barrier or areas less capable of producing inositol de novo. Further, because the PI signaling cascade is linked to muscarinic receptor/G_q protein activation, areas of the brain undergoing the highest rate of receptor activation should be preferentially affected by lithium. Indeed, previous studies in our laboratory lend support to these hypotheses (Watson and Lenox, 1996). Firstly, in studies of HN33 cells exposed to lithium, inositol supplementation (at concentrations as low as 5 μM) was able to prevent the down-regulation of MARCKS induced by 1 mM lithium, thereby supporting the inositol-depletion hypothesis. Secondly, addition of the muscarinic receptor agonist carbachol (1 mM) increased the MARCKS down-regulation observed (Watson and Lenox, 1996), which supports the notion that the action of lithium is augmented in cells or tissue regions undergoing the highest rate of receptor activation.

Whereas the mechanism of action of lithium has often been attributed to this modulation of PI signaling and its downstream effects, there is little evidence in support of a similar role for VPA; in fact, studies have indicated that VPA is probably not working through effects on PI signaling. In one study comparing the effects of lithium, carbamazepine, or VPA on IMPase activity, lithium and carbamazepine were found to decrease and increase IMPase activity, respectively, and VPA was shown to have no effect, at concentrations ranging from 2 μM to 5 mM (Vadnal and Parthasarathy, 1995). In another study, 2 mM valproate failed to increase accumulation of inositol phosphates, including the inositol monophosphates, inositol 1,3-bisphosphate, inositol 1,4-bisphosphate, and inositol 1,3,4-trisphosphate, whereas lithium exposure resulted in accumulation of all (Dixon and Hokin, 1997). In a study conducted in our laboratory, VPA failed to result in accumulation of CMP-PA (also known as CDP-DAG; see Figure 1.2), a metabolite of DAG and a sensitive measure of PI signaling, whereas lithium resulted in significant accumulation of CMP-PA in CHO-K1 and HN33 cells (Watson *et al.*, 1998). These data suggest that inhibitory effects on IMPase may not be the common site of action for mood-stabilizing agents. However, this does not dismiss the possibility that such agents may act indirectly or on alternate locations to alter the PI signaling cascade. Experiments discussed herein have been modeled after those described above for lithium, and will serve to clarify further the relationship between inositol or carbachol and VPA-induced effects. Based on data from previous studies showing a lack of influence by VPA on the PI signaling cascade, it is hypothesized that neither inositol nor carbachol will alter any observed effect of VPA on MARCKS protein expression.

As mentioned previously, attention will be given to the possible therapeutic relevance of findings. Inasmuch as lithium and VPA are commonly administered in combination to patients who fail to respond adequately to either drug alone, often resulting in amelioration or attenuation of manic symptoms, one study has been designed to test the hypothesis that there may be additive actions at the cellular level. Following

optimization of exposure conditions for each drug, cells will be exposed to the combination of VPA and lithium, and effects on MARCKS protein assessed. A final study involves exposing cells to a series of other psychotropic agents individually, in order to assess the specificity of action of mood-stabilizing agents and serve as a control for other studies conducted. If MARCKS mediates any of the mood-stabilizing properties of these agents, one would hypothesize that a variety of drugs efficacious in the treatment of bipolar disorder would elicit the MARCKS down-regulation observed following lithium exposure.

Experiments described thus far have simply focused on alterations in MARCKS protein expression following exposure to drug and manipulation of various other conditions *in vitro*. However, little is known of how these changes come about, or how MARCKS levels are regulated *in vivo*. Regulation of MARCKS expression is thought to occur in several ways: (1) tissue-specific expression, (2) developmentally regulated expression, (3) differentially regulated transcription rates, and (4) alterations in mRNA levels in response to PKC (Blackshear, 1993; Lobach *et al.*, 1993). VPA-induced effects on PKC itself will be discussed in detail in Chapter 5. Accumulating evidence shows that lithium and VPA have regulatory effects on various transcription factors, thereby suggesting a role for these mood stabilizers in altering gene expression. Studies in transfected cultured cells have indicated that VPA enhances expression of genes and reporters which contain AP-1 elements in their regulatory domains (Simon *et al.*, 1994; Kuntz-Simon *et al.*, 1995). Manji and colleagues showed further that VPA exposure resulted in a time- and concentration-dependent increase in DNA binding activity of the transcription factor AP-1 in both rat C6 glioma and human neuroblastoma (SH-SY5Y) cells; lithium reportedly induced similar effects (Chen *et al.*, 1997).

It is unknown how MARCKS expression is regulated beyond transcription, or how the lithium-induced down-regulation of MARCKS protein occurs. Expression of many proteins is known to be regulated not only by transcriptionally, but also by post-

transcriptional mechanisms, so that changes in mRNA stability play a crucial role in the control of GAP-43 expression, for example. Similar post-transcriptional mechanisms might also be at work in the case of MARCKS, and it is the purpose of these experiments to assess further this possibility. Preliminary studies in our laboratory have indicated that following lithium exposure, MARCKS mRNA levels do decline along a time course similar to that of the protein down-regulation (Watson and Lenox, 1997), and one would expect the same for VPA-induced alterations in MARCKS mRNA: a down-regulation of mRNA levels over time, at therapeutically relevant concentrations of VPA, and with a delayed onset of effect and protracted recovery of expression upon VPA withdrawal.

GAP-43

The growth-associated protein, GAP-43, is another major PKC substrate in brain which shares many similarities with MARCKS. Like MARCKS, GAP-43 binds both calmodulin and filamentous actin, albeit in a Ca^{++}-independent manner (reviewed in Benowitz and Routtenberg, 1997), and may play a role in exocytosis (Dekker *et al.*, 1989). GAP-43, like MARCKS, appears to be integral to normal nervous system development, because studies of mice lacking the protein as a result of antisense-mediated gene knockout show a high frequency of early postnatal death, with few animals surviving past three weeks of age and even fewer reaching maturity (Strittmatter *et al.*, 1995). Those mice that do survive exhibit abnormal neuronal pathfinding.

GAP-43 is a rapidly transported, presynaptic protein most highly expressed during development and post-injury regeneration, and immunohistochemistry and *in situ* hybridization studies have shown GAP-43 expression to be almost exclusively neuronal (Benowitz and Routtenberg, 1997). Neurite outgrowth is generally accompanied by a large increase in GAP-43 expression, and following differentiation and synaptogenesis, GAP-43 levels decline sharply and remain low, except in the most plastic regions of the

brain, such as associative and limbic areas, where intense immunostaining persists in adults throughout life. This selective expression is thought to confer the unique potential for ongoing structural remodeling (Benowitz and Perrone-Bizzozero, 1991).

GAP-43 is associated with the cytoskeleton and is concentrated in the growth cones of elongating axons and nerve terminals, thought to help stabilize growth cone morphology and promote growth cone adhesion (Aigner and Caroni, 1995). Support for this notion has been provided by a number of studies. Antisense-directed depletion of GAP-43 in growth cones results in the absence of spreading, branching, and adhesion of growth cones (and therefore, neurite outgrowth) in primary sensory neurons (Aigner and Caroni, 1993). Whereas suppression of GAP-43 expression has been found to yield adverse effects on axon outgrowth both *in vivo* and *in vitro* (Meiri *et al.*, 1998; Benowitz and Routtenberg, 1997; Strittmatter *et al.*, 1995; Jap Tjoen San *et al.*, 1992; Shea *et al.*, 1991), overexpression of the protein induced nerve sprouting in the adult nervous system of transgenic mice (Aigner *et al.*, 1995). Even in non-neuronal cells, expression of a GAP-43 transgene has been found to induce extensive process outgrowth accompanied by reorganization of the membrane cytoskeleton (Strittmatter *et al.*, 1994; Yankner *et al.*, 1990; Zuber *et al.*, 1989), yet, a mutant PC12 cell line which does not express GAP-43 still exhibits neurite outgrowth (Baetge and Hammang, 1991).

Although studies are in disagreement as to the precise role of GAP-43 in these events, it has been hypothesized that the almost exclusive presence of GAP-43 in neuronal cells, and the persistence of GAP-43 expression in highly plastic limbic regions, confers the ability to undergo the structural remodeling necessary for the functional plasticity of the brain (Benowitz and Perrone-Bizzozero, 1991). Indeed, Skene and Willard (1989) suggest that it is the induction of GAP expression that is necessary to post-injury remodeling, and that the inability of many areas of the CNS to induce high levels of GAP expression may in part underlie the failure of CNS axons to regenerate after axonal injury, whereas areas of the peripheral nervous system retain the ability to up-regulate GAP-43 as

well as to regenerate axons after injury. However, the presence of GAP-43 is probably not necessary to neurite outgrowth *per se*, but rather, plays an important role in regulating precisely the various parameters involved in the overall event, such as navigation or pathfinding (Aigner and Caroni, 1995). According to this "GAP hypothesis", nerve growth is controlled in part by the expression of certain growth associated proteins, such as GAP-43, which provide a mechanism for regulating neurite outgrowth and structural plasticity in the nervous system (Skene and Willard, 1989).

Because of the vital role of GAP-43 in effecting plastic changes in brain, experiments have been designed to address the effect(s) of VPA exposure on GAP-43 expression in HN33 cells. It may be that part of the efficacy of VPA in either bipolar disorder or epilepsy, or both, lies in its ability to elicit structural modifications of plastic regions exhibiting the pathology which leads to the manifestation of the particular disorder. Indeed, Benowitz and Routtenberg (1997) pointed out that in brain of humans and other primates, the highest levels of GAP-43 expression occur in regions known to be involved with higher-level associative processes, lending support to the notion that GAP-43 is important for maintaining plasticity.

Whereas *in vitro* studies in HN33 cells have shown no evidence for regulation of GAP-43 by lithium, neither has lithium been shown to alter the morphology of these cells. Preliminary studies have suggested that chronic VPA exposure induces a morphological alteration in these cells which might be further characterized as differentiation (see Chapter 5). Since neurite outgrowth and neuronal differentiation are known to be associated with up-regulation of GAP-43 protein expression, it will be of interest to investigate the effect of VPA on this protein.

The results of these studies will be instructive, for in addition to the development of a model which may help explain the role of MARCKS and other plasticity proteins (such as GAP-43) in the mechanism of action of both VPA and lithium, findings will contribute to a better understanding of the pathophysiology involved in bipolar disorder.

The notion of synaptic remodeling as the underlying means for the drug- or therapy-induced stabilization of mood disorders has been supported by some reports. One study found increased levels of neural cell adhesion molecule (N-CAM)-immunoreactive proteins in the cerebrospinal fluid of a population of bipolar patients (Poltorak *et al.*, 1996). Abnormal function or turnover of N-CAM, which plays a role in synaptogenesis, morphogenesis, and plasticity of the nervous system, may be suggestive of abnormal synaptic plasticity in affected individuals. With regard to drug therapy for this disorder, not only do lithium and VPA affect expression of proteins involved in neural plasticity and cellular response (as previously discussed), but both drugs have also been shown to have effects on transcriptional factors which regulate gene expression, and it is known that long-term changes in neuronal synaptic function are dependent upon the selective regulation of gene expression (Manji and Lenox, 1994). Lamotrigine, another drug used in treatment of bipolar disorder, was recently shown to alter neuronal hyperexcitability via inhibition of both Na^{+} and Ca^{++} channels, findings which were interpreted by the authors to signify modification of synaptic plasticity (Xie and Hagan, 1998). Such evidence is indirect, but overall suggestive that changes in synaptic plasticity may be a mechanism by which anti-bipolar (anti-manic and mood-stabilizing) therapy facilitates long-term changes in affect.

Results

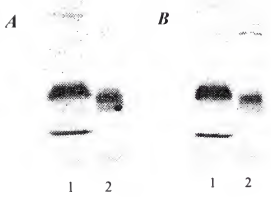


Figure 3.1. MARCKS protein. Shown are Western immunoblots comparing MARCKS protein expression in (1) mouse brain and (2) HN33 cells. *A* shows cytosolic MARCKS expression, and *B* shows membrane-associated MARCKS.

Comparative MARCKS expression in HN33 cells (used in this study) vs. mouse brain is shown in Figure 3.1 following enhanced chemiluminescent (ECL) detection. Differences in apparent molecular weight may result from differential phosphorylation or myristoylation of the protein. Note the more pronounced MARCKS protein expression in mouse brain, even with comparable amounts of total loaded protein.

Effect of VPA Exposure on MARCKS Protein in HN33 Cells

VPA was administered at concentrations ranging from 0.001 to 1.0 mM, for 1 or 3 d (Figures 3.2 and 3.3, respectively). Representative Western blots are presented, along with quantitation of mean data. Significant concentration- and time-dependent effects were observed. Chronic (3 d) VPA exposure results in a concentration-dependent reduction in MARCKS levels, with a more pronounced reduction in membrane than

soluble fraction of these cells. Significant down-regulation of MARCKS is observed in the membrane fraction at concentrations as low as 0.06 mM ($p<0.005$), and in the soluble fraction at 0.1 mM and higher concentrations ($p<0.005$). These concentrations correlate well with the target therapeutic serum concentration range used clinically for treatment of bipolar disorder.

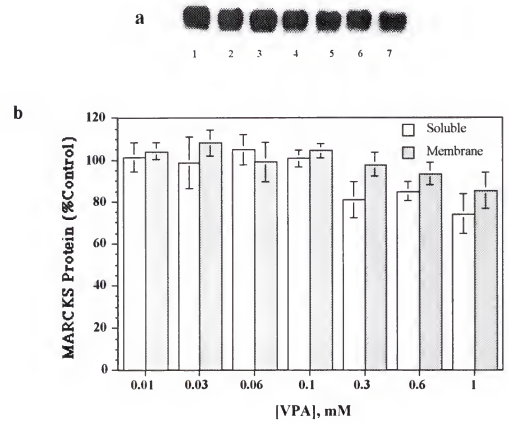


Figure 3.2. Concentration-dependent down-regulation of MARCKS protein in HN33 cells following 1 d VPA exposure. (a) Representative Western blot of membrane-associated MARCKS expression following 1 d VPA exposure; mean data are depicted graphically in 3.2b. Lanes 1-7 were treated for 1 d as follows: untreated control, 0.01 mM, 0.03 mM, 0.1 mM, 0.3 mM, 0.6 mM, and 1.0 mM VPA, respectively. (b) This figure shows quantitatively the MARCKS protein levels in HN33 cells following 1 d exposure to varying concentrations of VPA. Data, expressed as percent of MARCKS compared to untreated control cells grown in parallel, were derived from Western immunoblots (e.g., Figure 3.2a) and are the mean of at least four determinations. Error bars represent S.E.M.

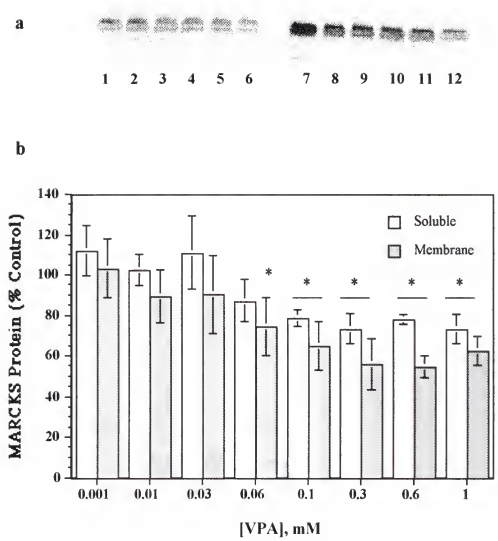


Figure 3.3. Concentration-dependent down-regulation of MARCKS protein in HN33 cells following 3 d VPA exposure. (a) Representative DAB Western blot of MARCKS expression following 3 d VPA exposure. Lanes 1-6 show soluble MARCKS following 3 d treatment with 0, 0.03, 0.06, 0.1, 0.3, and 0.6 mM VPA, respectively, and lanes 7-12 show membrane MARCKS following exposure to the same doses. (b) This figure shows quantitative data for mean MARCKS levels following 3 d VPA exposure. Data, expressed as percent of MARCKS compared to untreated control cells grown in parallel, were derived from Western immunoblots and are the mean of at least four determinations. Error bars represent S.E.M. *$p<0.005$

Time Course of VPA-Induced MARCKS Down-Regulation in HN33 Cells

HN33 cells were exposed continuously to 0.6 mM VPA for periods ranging from 0 to 7 d. This concentration is equivalent to 100 ng/ml, which is within the reported therapeutic range for the mood stabilizing action of VPA (50-125 ng/ml). Cells were observed to undergo a significant down-regulation of MARCKS protein expression in both fractions as early as 3 d, with no effect on MARCKS at 1 d (see Figure 3.4). The MARCKS reduction in the membrane fraction is more pronounced than that in the cytosol. Three d, 5 d, and 7 d values are significantly different ($p<0.01$) from 1 d MARCKS values in both soluble and membrane fractions of these cells. These data are consistent with the clinical profile of this drug, which exhibits a delay in onset of antimanic action at target therapeutic concentrations.

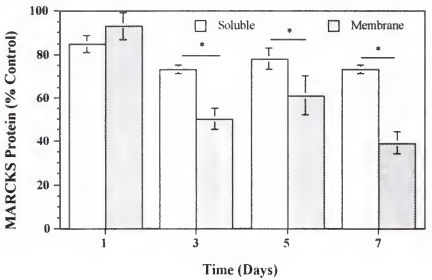


Figure 3.4. Time course of VPA-induced MARCKS down-regulation in HN33 cells. This figure illustrates the time-dependency of VPA-induced MARCKS protein down-regulation. HN33 cells were exposed to 0.6 mM VPA, a clinically relevant therapeutic concentration, for 1, 3, 5, or 7 d, and MARCKS expression was assessed by quantitative immunoblot analysis. Results, expressed as percent of untreated controls, represent the mean ± S.E.M. of at least four determinations. *$p<0.01$

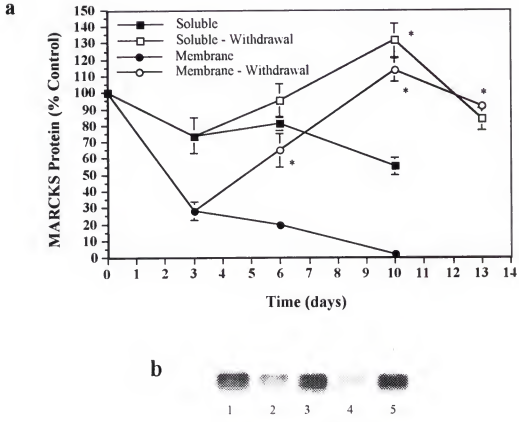


Figure 3.5. Recovery of MARCKS following VPA-induced long-term down-regulation. (a) This figure shows the time course of VPA-induced MARCKS down-regulation in HN33 cells, as well as the recovery of MARCKS protein to basal levels following drug withdrawal. HN33 cells were exposed to 0.6 mM VPA for 3 d, at which time the drug was either removed or treatment continued. MARCKS levels were assessed through Western immunoblot (Figure 3.5b is representative), and results are expressed as percent of untreated control cultures grown in parallel. Results represent mean ± S.E.M. of at least three determinations. *$p<0.0001$ (b) Shown is a representative Western blot of MARCKS expression in the membrane fraction of HN33 cells following chronic VPA exposure and subsequent withdrawal. Lanes 1 and 3 show control levels of MARCKS protein. Lane 2 shows MARCKS expression following 3 d VPA exposure (compare to Lane 1), and Lane 4 shows MARCKS expression following 6 d VPA exposure (compare to Lane 3). Lane 5 represents a VPA withdrawal/recovery group, and shows MARCKS in cells exposed continuously for 3 d to 0.6 mM VPA, and then for 3 d to media free of drug.

Recovery of MARCKS following VPA-Induced Long-Term Down-Regulation

As shown in Figure 3.5a, HN33 cells exposed to 0.6 mM VPA for 0 to 14 d exhibited a significant down-regulation of MARCKS protein expression in both fractions

(membrane > soluble) over time, as previously demonstrated in Figure 3.4. After 3 d of continuous VPA exposure and subsequent drug washout and withdrawal, MARCKS protein levels recovered with significant increase ($p<0.0001$) to control levels within 3-7 d of culture in drug-free medium (Figure 3.5a). A representative Western blot is shown in Figure 3.5b. These findings are consistent with clinical evidence for delayed onset of anti-manic action of VPA, as well as prolonged anti-manic efficacy following drug discontinuation. It is difficult to discern whether this experimental return of MARCKS to baseline levels is the result of either a shift in the kinetics of MARCKS production/degradation, or due to repopulation of the *in vitro* environment with MARCKS-replete daughter cells.

Effects of Carbachol and *myo*-Inositol on the VPA-Induced Down-Regulation of MARCKS

In order to examine the role of PI signaling in the mechanism of action of this drug's effects on MARCKS, cells were exposed to VPA alone or in combination with either carbachol (1 mM) or *myo*-inositol (up to 1 mM). In contrast to previous results obtained with LiCl (Watson and Lenox, 1996), the VPA-induced down-regulation of MARCKS protein expression was not altered by addition of carbachol (Table 3.1 and Figure 3.6) to the culture medium. Similarly, supplementation of *myo*-inositol (Table 3.2 and Figure 3.7) to the culture medium at concentrations up to 1 mM did not modify the VPA-induced down-regulation of MARCKS, whereas the LiCl-induced down-regulation was prevented by the addition of as little as 5.0 μM *myo*-inositol. Representative Western blots are presented in Figures 3.6 and 3.7. It is difficult to rule out completely the role of PI signaling based on the data in Table 3.1 alone, due to the maximal level of MARCKS down-regulation achieved in the case of VPA. However, the data in Table 3.2 are more conclusive, as the levels of MARCKS reduction elicited by VPA and lithium are more comparable.

Table 3.1. Addition of Carbachol Fails to Potentiate the VPA-Induced MARCKS Down-Regulation in HN33 Cells. HN33 cells were exposed to 0.6 mM VPA for 3 d or 1 mM lithium for 7 d, in the presence or absence of the muscarinic agonist carbachol. Results are presented as percent of untreated control cultures grown in parallel, and represent the mean ± S.E.M. of at least three determinations. *p<0.0001

	MARCKS Protein, % Control (Mean ± S.E.M.)			
	VPA-Exposed		LiCl-Exposed	
[Carbachol]	Soluble	Membrane	Soluble	Membrane
0 mM	41.8 ± 6.6	17.0 ± 8.5	81.6 ± 4.5	90.2 ± 3.1
1 mM	46.5 ± 7.5	10.8 ± 2.0	66.6 ± 6.1**	68.7 ± 5.9**

Figure 3.6. Effect of carbachol on VPA-induced MARCKS down-regulation. Shown is a representative Western blot of MARCKS expression following VPA exposure with or without the concomitant addition of carbachol. Lane 1 shows membrane-associated MARCKS levels in untreated control HN33 cells, Lane 2 shows MARCKS in 3 d VPA-treated cells, Lane 3 shows MARCKS in carbachol-treated cells, and Lane 4 shows MARCKS in cells treated concomitantly with VPA and carbachol. Mean data are reported in Table 3.1.

Table 3.2. Inositol Supplementation Fails to Attenuate the VPA-Induced MARCKS Down-Regulation in HN33 Cells. HN33 cells were exposed to 0.6 mM VPA for 3 d or 1 mM lithium (+1 mM carbachol) for 7 d, in the presence (or absence) of varying concentrations of *myo*-inositol. Results are presented as percent of untreated control cultures grown in parallel, and represent the mean ± S.E.M. of at least three determinations (N.D. = not determined). *p<0.005

	MARCKS Protein, % Control (Mean ± S.E.M.)			
	VPA-Exposed		LiCl/carbachol-Exposed	
[*myo*-Inositol]	Soluble	Membrane	Soluble	Membrane
0	83.0 ± 6.0	57.0 ± 5.0	66.6 ± 6.1	68.7 ± 5.9
0.5 μM	N.D.	N.D.	71.1 ± 4.8	68.4 ± 4.4
5 μM	N.D.	N.D.	97.9 ± 3.6*	104.3 ± 5.3*
40 μM	77.8 ± 2.4	54.8 ± 5.5	98.5 ± 7.5*	103.6 ± 8.2*
1 mM	78.3 ± 9.4	51.3 ± 10.8	N.D.	N.D.

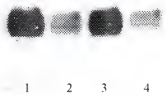


Figure 3.7. Effect of inositol on VPA-induced MARCKS down-regulation. Shown is a representative Western blot of MARCKS expression following VPA exposure with or without concomitant addition of *myo*-inositol. Lanes 1-4 show membrane-associated MARCKS in (1) control cells, (2) 3 d 0.6 mM VPA-treated cells, (3) inositol-treated cells, and (4) combined VPA- and inositol-treated cells. Mean data are reported in Table 3.2.

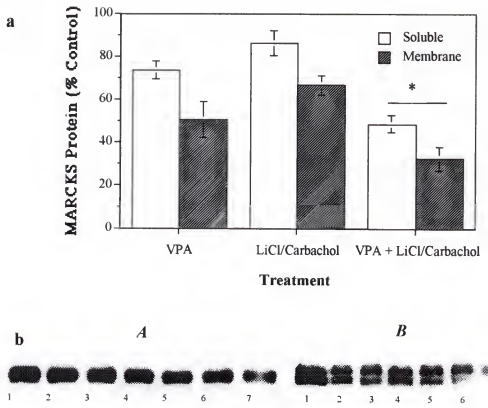


Figure 3.8. Additive effect of combined VPA and lithium exposure on MARCKS protein expression. (a) HN33 cells were exposed to 0.6 mM VPA and/or 1 mM lithium (+ 1 mM carbachol) in inositol-free DMEM for 3 d. MARCKS protein levels, expressed as percent of untreated control, were assessed by Western immunoblot analysis (see Figure 3.8b), and values represent mean ± S.E.M. of at least three determinations. *$p<0.01$ (b) Representative Western blot of MARCKS protein following 3 d exposure of cells to VPA and/or lithium. *A* shows cytosolic MARCKS in HN33 cells after the following treatments: no drug (1); 0.3 mM VPA (2); 0.6 mM VPA (3); 1 mM lithium (4); 1 mM lithium + 1 mM carbachol (5); 1 mM lithium + 1 mM carbachol + 0.3 mM VPA (6); and 1 mM lithium + 1 mM carbachol + 0.6 mM VPA (7). *B* shows MARCKS in the membrane fraction of HN33 cells following the same treatments for 3 d.

Additive Effect of Combined VPA and LiCl Exposure on MARCKS Protein Expression in HN33 Cells

HN33 cells were exposed to 0.6 mM VPA and/or 1 mM LiCl/1 mM carbachol for a period of 3 d, in order to assess the effects of the VPA-lithium combination on MARCKS protein expression and distribution. Results, presented in Figure 3.8a, show that a greater reduction of MARCKS protein was observed in both soluble and membrane

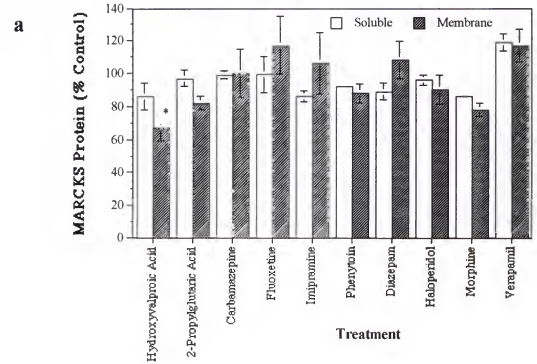
fractions of cells exposed to both VPA and lithium/carbachol than was produced by either VPA or lithium/carbachol alone. The enhanced reduction in MARCKS protein produced by the combination of VPA and lithium/carbachol at these concentrations appeared to be additive. Furthermore, the reduction in MARCKS protein produced by the combined treatment of VPA and lithium/carbachol was greater in the membrane fraction than in the soluble fraction. A representative Western blot is shown in Figure 3.8b.

Effects of Other Psychotropic Agents on MARCKS Protein Expression

Eight structurally and functionally distinct psychotropic drugs (carbamazepine, fluoxetine, imipramine, phenytoin, diazepam, haloperidol, morphine, and verapamil) were administered to HN33 cells for 1, 3, or 7 d, and their effects on MARCKS protein expression were assessed. Drugs were chosen to represent a wide range of psychopharmacological actions. Minimal drug concentrations were based on the therapeutically effective plasma concentration for each drug in humans, as outlined in Table 3.3. Additionally, cells were exposed to concentrations up to 20 times the highest reported therapeutic concentration so as not to overlook potential effects at supra-therapeutic doses. At each concentration tested, cell viability, growth rate, and gross morphological appearance of HN33 cells exposed to each of the psychotropic drugs were not notably different than those of cells grown in the absence of drug. Data obtained following 3 d exposure are shown in Figure 3.9a. For all of the drugs listed, no significant dose effect on MARCKS protein expression was measured at any of the concentrations or time points tested.

MARCKS protein levels in HN33 cells were also measured after exposure to each of two VPA analogues or metabolites, hydroxyvalproic acid (HVPA, or 2-n-propyl-5-hydroxy-pentanoic acid) and 2-n-propylglutaric acid (2-PGA), for 3 or 7 d (Table 3.3; Figure 3.9a,b) These VPA metabolites, the structures of which are depicted in Figure 3.9c, were provided by Abbott Laboratories (Abbott Park, IL), and have been found to

have little or no anticonvulsant efficacy *in vivo* (Chapman *et al.*, 1982; J. Sullivan, personal communication). Cells were exposed to concentrations of 0.6, 1.5, and 3.0 mM. Unlike VPA, which produced noticeable changes in cell morphology at 0.6 mM (see Chapter 6), these analogs produced no such change at this or higher concentrations. Following HVPA exposure, no significant reduction in MARCKS protein was evident in the soluble fraction, even at concentrations 5-10 times higher than the minimal effective concentration of VPA. However, in the membrane fraction, a significant reduction in MARCKS protein was observed only in cells exposed to 3.0 mM HVPA ($p<0.01$ at 3 d; $p<0.05$ at 7 d). At this highest concentration tested (3.0 mM; 5X the therapeutic level of VPA), a 30-35% reduction in MARCKS protein was observed following 3-7 d exposure. Following 2-PGA exposure, no significant reduction in MARCKS protein was apparent in the soluble fraction, even at concentrations 5-10 times higher than the minimal effective concentration of VPA. A small but insignificant reduction in MARCKS protein in the membrane fraction was observed following 3.0 mM exposure to 2-PGA for 3-7 d. A representative Western blot is presented in Figure 3.9b.



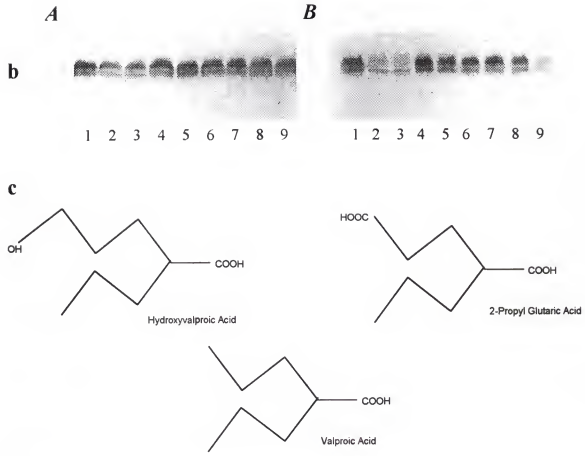


Figure 3.9. Effects of other psychotropic agents on MARCKS protein expression. (a) This figure shows the relative lack of effect of various other psychotropic agents on MARCKS protein expression in HN33 cells. Cells were exposed to drugs other than VPA or lithium (see Table 3.3 for details), as follows: 3.0 mM hydroxyvalproic acid, 7 d; 3.0 mM 2-propylglutaric acid, 7 d; 100 μM carbamazepine, 7 d; 1 μg/ml fluoxetine, 3 d; 1 μg/ml imipramine, 7 d; 20 μg/ml phenytoin, 3 d; 1 μg/ml diazepam, 7 d; 20 ng/ml haloperidol, 7 d; 20 μg/ml morphine, 7 d; 50 ng/ml verapamil, 7 d. MARCKS levels were assessed as previously described; data represent mean ± S.E.M. of at least three determinations. *$p<0.05$ (b) MARCKS expression following exposure to VPA and two metabolites. Shown is a representative Western blot of MARCKS protein in HN33 cells after 7 d exposure to the following: no drug (1); 0.6 mM VPA purchased from Sigma Chemical Company (2); 0.6 mM VPA provided by Abbott Laboratories (for comparison purposes; 3); 0.6 mM (4), 1.5 mM (5), and 3.0 mM (6) 2-propylglutaric acid; 0.6 mM (7), 1.5 mM (8), and 3.0 mM (9) hydroxyvalproic acid. *A* shows cytosolic MARCKS expression, and *B* shows membrane-associated MARCKS. (c) Structural comparisons of VPA and metabolites. Shown are the chemical structures of VPA and two of its metabolites, hydroxyvalproic acid, or 2-n-propyl-5-hydroxy-pentanoic acid, and 2-n-propylglutaric acid.

Table 3.3. Drugs, Sources, and Exposure Conditions for HN33 Cells.
This table lists the drugs to which HN33 cells were exposed, their sources, target circulating (plasma) concentrations in humans, concentrations tested in HN33 cells, and vehicle in which drug was suspended. Drug concentrations tested encompassed a broad range, including supratherapeutic concentrations, in order to account for discrepancies, such as those presented by variation in circulating serum vs. brain concentrations of drug.

Drug / Source	Target Plasma Conc. in Humans	Concentrations Tested	Vehicle
Sodium Valproate Sigma #P-4543 Abbott #A-44089.5	50-125 μg/ml = 0.3-0.9 mM (seizure control)[a,b]	0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1.0 mM	dH_2O
2-Propylglutaric Acid Abbott #A-49999.0		0.6, 1.5, 3.0 mM	dH_2O
Hydroxyvalproate, sodium salt Abbott #A-49822.5		0.6, 1.5, 3.0 mM	dH_2O
Carbamazepine Sigma #C-4024	8-12 μg/ml = 33-50 μM (seizure control)[b,c]	10, 25, 100 μM	ETOH
Fluoxetine-HCl Lilly #110140L/F08083	varies	50, 200, 1000 ng/ml	dH_2O
Imipramine-HCl Sigma #I-7379	100-300 ng/ml (antidepressant)[a]	50, 200, 1000 ng/ml	dH_2O
Phenytoin, sodium salt Sigma #D-0931	10-20 μg/ml (anticonvulsant)[d,a]	1, 10, 20 μg/ml	ETOH
Diazepam Sigma #D-0899	300-400 ng/ml (anxiolytic)[a,b] >600 ng/ml (seizure control)[b]	50, 200, 1000 ng/ml	ETOH
Haloperidol Sigma #H-1512	4-20 ng/ml (anti-psychotic)[b]	5, 20, 100 ng/ml	dH_2O
Morphine Sulfate Sigma #M-8777	5-20 ng/ml (analgesic in non-tolerant patients)[c]	5, 20, 100 ng/ml	dH_2O
Verapamil-HCl Sigma #V-4629	varies	50 ng/ml	ETOH

[a]Holford and Benet, 1995; [b]Benet and Williams, 1990; [c]Weiner, 1996; [d]Porter and Meldrum, 1995.

Effect of VPA on MARCKS mRNA

HN33 cells were exposed to 0.6 mM VPA for 0 to 14 d, and MARCKS mRNA levels were measured by RPA as described in Materials and Methods. As shown in Figure 3.10a, MARCKS mRNA levels decrease over time with continual VPA exposure ($p<0.05$). A representative RPA is shown in Figure 3.10b.

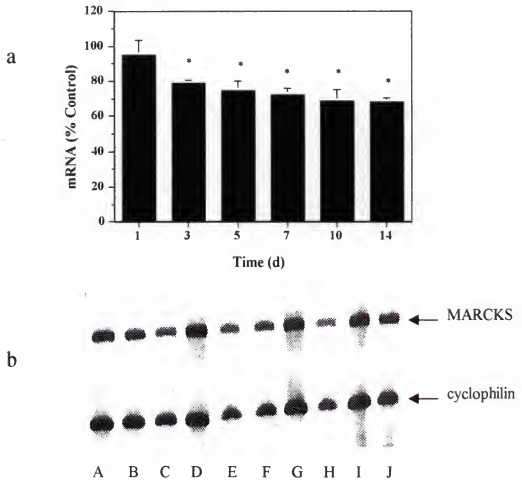


Figure 3.10. Time course of VPA-induced MARCKS mRNA alterations. (a) This figure shows the time course of VPA-induced alterations in MARCKS mRNA. HN33 cells were exposed to 0.6 mM VPA for 0-14 d, and MARCKS mRNA levels were assessed by RPA, with the aid of a cyclophilin internal control, as described in Materials and Methods. Results for each treatment are calculated as a ratio of MARCKS to cyclophilin, and then each value is expressed as a percentage (± S.E.M.) of untreated, control samples prepared in parallel. *$p<0.05$ Shown in (b) is a representative RPA. Upper band reflects MARCKS mRNA, and lower band reflects cyclophilin mRNA. Treatments are as follows: A, control; B, 1 d VPA; C, 3 d VPA; D, control; E, 5 d VPA; F, 7 d VPA; G, control; H, 10 d VPA; I, control; J, 14 d VPA.

Effect of VPA on GAP-43 Expression in HN33 Cells

GAP-43 expression in HN33 cells is compared to its expression in rat brain in Figure 3.11. Following 3 d, 0.6 mM VPA exposure, GAP-43 levels in HN33 cells increase, as shown in Figure 3.12. This increase is concentration-dependent, as shown in Figure 3.13 ($p<0.01$). HN33 cells were exposed for 3 d to concentrations of VPA ranging from 0.01 mM to 1.0 mM, and membrane-associated GAP-43 expression is shown (Figure 3.13).

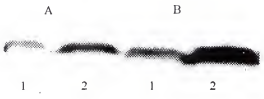


Figure 3.11. GAP-43. Shown is a Western immunoblot comparing GAP-43 expression in (1) soluble and (2) membrane fractions of (A) HN33 cells and (B) rat brain.

Figure 3.12. Effect of chronic VPA exposure on GAP-43 expression in HN33 cells. Shown is a representative Western blot of GAP-43 expression in the membrane fraction of HN33 cells following VPA exposure. Lane 1 shows GAP-43 expression in untreated control cells, and Lane 2 shows GAP-43 in cells treated for 3 d with 0.6 mM VPA.

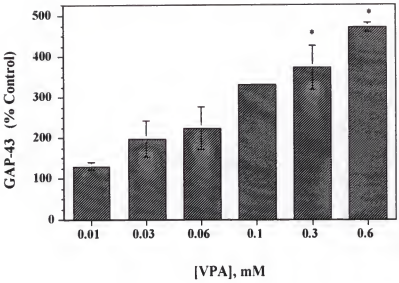


Figure 3.13. Concentration-dependent increase in GAP-43 expression in HN33 cells following VPA exposure. HN33 cells were exposed for 3 d to varying concentrations of VPA, and membrane-associated GAP-43 expression was assessed by Western immunoblot analysis. Results represent the mean of two or three determinations, and are expressed as the percent of GAP-43 protein expressed in untreated control cultures grown in parallel. *p<0.01

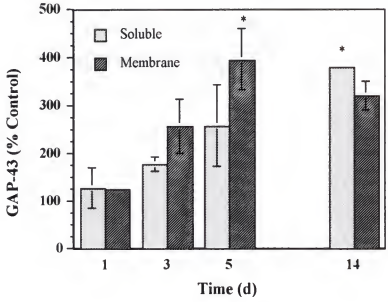


Figure 3.14. Time course of VPA-induced GAP-43 increase in HN33 cells. HN33 cells were exposed to 0.6 mM VPA for times ranging from 1 d to 14 d, and GAP-43 levels were assessed by Western immunoblot analysis. Results reflect the mean of at least three determinations, and are expressed as percent of GAP-43 in an untreated control. *p<0.05

Time Course of VPA-Induced GAP-43 Increase in HN33 Cells

HN33 cells were exposed to 0.6 mM VPA for 0-14 d; results are shown in Figure 3.14. Up-regulation of GAP-43 was observed in membrane and soluble fractions of cells exposed to VPA for 5 d and 14 d, respectively ($p<0.05$). Because of the experimental protocol, which entailed growing cells to confluency and then diluting them every 3-4 d, the data may not reflect the full potential for GAP-43 induction, since each splitting and re-seeding procedure involved trypsinization and potential disruption of cellular processes.

Discussion

HN33 cells were utilized as the model for all studies, because their morphological, immunological, and electrophysiological characteristics resemble those of hippocampal neurons in culture. HN33 cells were chosen as the *in vitro* cell model in which to investigate multiple steps in the PKC/GAP-43/MARCKS pathway, because of their relative homogeneity and the presence of signal transduction pathways of interest. This is in contrast to the use of other models, such as primary neuronal cultures, which are more neurochemically heterogeneous in nature. In primary cultures, for example, it would be difficult to assess whether PKC and other messenger systems were affected in the same neuron. The homogeneity of HN33 cells makes signaling research and results less confounding.

MARCKS

VPA exposure elicits both a time- and concentration-dependent reduction in MARCKS protein expression. It has been well established that the clinical antimanic response to VPA requires a lag period of days to weeks following drug administration

before onset of action, although loading strategies have proven useful in achieving faster response (within 3 d) (Bowden *et al.*, 1994; McElroy *et al.*, 1989). Consistent with such clinical observations, a significant reduction of MARCKS protein in both soluble and membrane fractions of HN33 cells was not observed following acute (1 d) VPA administration, except at levels above the maximal target therapeutic serum concentration (1 mM). At 0.6 mM VPA, the down-regulation of MARCKS was greatest between d 0 and d 3, and little further reduction was observed upon continued exposure (5-10 d). With regard to therapeutic concentrations, there have been reports of clinical response at plasma concentrations of VPA as low as 45 μg/ml (Bowden *et al.*, 1994), which is comparable to the observed effects of VPA on MARCKS down-regulation at concentrations as low as 0.1 mM in this study. However, VPA is generally thought to be most effective at therapeutic concentrations of 50-125 μg/ml (0.3-0.75 mM), where the most robust effect of VPA on MARCKS was observed in the HN33 cells. Following VPA-induced MARCKS down-regulation and subsequent drug withdrawal, MARCKS levels in these cells recovered to baseline within 3-7 d. This finding is consistent with the third major parameter discussed with regard to clinical relevance, that of long-term effect of VPA and delayed return of clinical response to baseline following treatment discontinuation.

With regard to combined VPA/lithium exposure, a greater reduction of MARCKS protein was observed in cells following exposure to the combination than was produced by either VPA or lithium/carbachol alone, and the effects of the two drugs appear to be approximately additive. Whereas VPA induced a mean reduction in MARCKS expression of 26% in the soluble fraction and 50% in the membrane, and lithium/carbachol elicited mean reductions of 14% and 33% in soluble and membrane, respectively, the combination of VPA plus lithium/carbachol resulted in a 51% drop in soluble MARCKS and a 67% drop in membrane-associated MARCKS. These data are in agreement with clinical studies which show VPA/lithium combination therapy to have greater therapeutic efficacy for

treatment of bipolar disorder than either agent administered individually (Calabrese and Delucchi, 1989; Hayes, 1989). In contrast to lithium, which exerts a preferential down-regulation of MARCKS in the soluble fraction of HN33 cells (Watson and Lenox, 1996), the effect of VPA on MARCKS was most pronounced in the membrane fraction of these cells. While the significance of this finding is unclear, the differences observed in the patterns of MARCKS down-regulation between these two drugs may be a function of differences in the pathways of PKC activation, as later discussed. This is of particular interest in light of the findings that concomitant exposure of cells to both VPA and lithium appears to have an additive effect on MARCKS down-regulation at therapeutic concentrations of both drugs, and such findings support the notion that the two drugs operate through separate mechanisms.

In contrast to lithium, which appears to rely on relative depletion of *myo*-inositol and receptor activation of PI signaling to optimize its effect on MARCKS in the immortalized hippocampal cells, these manipulations had no effect on the VPA-induced down-regulation of MARCKS in these cells. Addition of carbachol or inositol (even at mM concentrations) to the culture medium had no effect on the VPA-induced reduction in MARCKS, and these findings are consistent with the lack of effect of VPA on receptor-coupled accumulation of CMP-PA in the CHO-K1 cells (Watson *et al.*, 1998). CMP-PA, also known as CDP-DAG, is a byproduct of DAG metabolism and precursor for PI reformation, and serves as a sensitive measure of cycling through the PI signaling cascade (refer to Figure 1.2). Thus, in spite of the shared property of lithium and VPA in down-regulating the expression of MARCKS, these data suggest that the mechanism through which this occurs may involve different pathways. Nonetheless, there appears to be evidence for a role of PKC regulation in the action of both lithium and VPA, for previous studies have demonstrated that phorbol esters down-regulate MARCKS protein in neuronally derived cell populations in a PKC-dependent manner (Watson *et al.*, 1994). This will be further discussed in later chapters.

Two structural analogs of VPA (see Figure 3.9c), which are significantly less potent as anti-convulsants in comparison to VPA (Chapman *et al.*, 1982), were studied. Both analogs are known metabolites of VPA, although they are produced through minor metabolic pathways and represent a minimal percentage of total brain plasma concentration (Chapman *et al.*, 1982; Eadie, 1991). In previous studies, neither hydroxyvalproic acid nor 2-propylglutaric acid was shown to be effective in preventing pentylenetetrazol-induced seizures in mice (Chapman *et al.*, 1982; J. Sullivan, personal communication), and neither agent has been tested clinically for efficacy in the treatment of bipolar disorder. In our studies, hydroxyvalproic acid produced a statistically significant effect in down-regulating membrane-associated MARCKS, but at a concentration well above that observed for VPA (3.0 mM hydroxyvalproic acid vs. 0.1-1.0 mM VPA). Comparatively, the 2-propylglutaric acid analog possessed little potency in down-regulating MARCKS in either the membrane or cytosolic fraction. Future analysis of structural differences among these VPA compounds may prove useful in identifying structurally related fatty acids that may be more or less efficacious not only in down-regulating MARCKS, but also potentially as mood stabilizers.

Exposure of immortalized hippocampal cells to structurally and functionally diverse psychotropic agents, including carbamazepine, fluoxetine, imipramine, phenytoin, diazepam, haloperidol, morphine, and verapamil, did not result in a down-regulation of MARCKS, even at doses that were well above the therapeutic range. It is of particular note that carbamazepine, another anti-convulsant used less frequently in the treatment of manic patients, and verapamil, also used unconventionally and perhaps less efficacious than the more established treatments (Janicak *et al.*, 1998; Walton *et al.*, 1996; Mathis *et al.*, 1988; Barton and Gitlin, 1987), did not have any effect on MARCKS expression. Carbamazepine has been shown to have effects within the adenylyl cyclase cascade, a property shared by lithium at higher concentrations (Manji *et al.*, 1995; Mork *et al.*, 1992). However, carbamazepine has generally been shown to be less effective overall than

lithium or VPA as a mood stabilizer (Keck *et al*., 1992; Bowden, 1996), and verapamil, a calcium channel blocker, has yet to be studied extensively for this application.

Following exposure to a therapeutic serum concentration (0.6 mM) of VPA for up to 14 d, HN33 cells exhibited decreased MARCKS mRNA levels, along a time course similar to that of observed MARCKS protein alterations. This finding suggests that the down-regulation of MARCKS protein might be attributed, at least in part, to a reduction of its precursor mRNA template. With regard to MARCKS regulation, such evidence may support more than one hypothesis. The observed reduction in MARCKS protein may be the result of either altered transcriptional rates, or post-transcriptional regulation, via decreased mRNA stability, decreased translation, or increased degradation. Yet, the observed alterations in mRNA expression support the notion that MARCKS is being regulated at the level of mRNA synthesis or stability. Recent studies in our lab have shown that lithium, while altering both MARCKS mRNA and protein levels, did not affect mRNA stability (Watson and Lenox, 1997); this suggests that the lithium-induced alteration in MARCKS mRNA is primarily attributable to effects on transcription of the MARCKS gene.

These studies suggest that MARCKS may serve as a target in the brain for the selective action of mood stabilizing drugs. Although the therapeutic action of these drugs can probably not be attributed fully to their effects on MARCKS, it is plausible that the role of MARCKS in modulating plasticity may account for some of the long-term stabilizing actions exhibited by VPA and lithium in the treatment of bipolar disorder. This notion is supported by the finding that other drugs were set apart by both their inability to down-regulate MARCKS as well as their inefficacy in treating bipolar disorder. Further support for the notion of selective action in the CNS has been provided by data from our laboratory which shows that the expression of MARCKS protein in two separate murine macrophage cell lines (IC-21 and MH-S) is unaltered following chronic exposure to therapeutic levels of VPA (3-7 d at 0.6 mM; data not shown). In addition to effects of

VPA on MARCKS, such alterations in neuronal plasticity may result from actions on other potential protein substrates, as discussed below.

GAP-43

It is clear from these studies that VPA has a time- and concentration-dependent effect on GAP-43 expression in HN33 cells. Further, the concentration range over which this increase in GAP-43 occurred was comparable to the concentration range over which the down-regulation in MARCKS protein occurred, and this concentration range is well within the target therapeutic serum concentration. There are several potential implications of such an increase in GAP-43 expression, since GAP-43 is an early marker of post-mitotic neurons, and is highly expressed during axonal growth and synapse formation (Aigner *et al.*, 1995). For example, increased GAP-43 expression might signify synaptic regrowth and/or remodeling, cytoprotection, or even development of aberrant pathways. Synaptic remodeling was discussed previously as a potential mechanism for long-term mood stabilization. A novel finding observed in carrying out these studies was that following chronic (>1 d) VPA exposure, HN33 cells appeared to become more adherent to the tissue culture flasks in which they were grown. This is of interest in light of the finding that chronic VPA exposure also elicited an increase in expression of GAP-43, which is known to be important for promoting and maintaining growth cone adhesion during neurite outgrowth (Aigner and Caroni, 1995). Later chapters will include further studies on the growth and morphological changes induced by VPA.

As previously mentioned, preliminary studies have shown no evidence for lithium-induced regulation of GAP-43 expression in HN33 cells, and this major difference between VPA and lithium is intriguing. Since the two drugs share similar effects on MARCKS, one might hypothesize that this effect provides a basis for their common ability to treat bipolar disorder. Conversely, the difference in their ability to affect GAP-43 might

relate either to their differing efficacies in treating various types of bipolar disorders (i.e. VPA is generally more efficacious in treating rapid-cycling bipolar disorder), or, alternatively, to their very different pharmacological profiles aside from their mood-stabilizing efficacy (i.e., VPA is also an anti-epileptic, whereas lithium is not).

Conclusions

With regard to alterations in protein expression and their necessity and/or sufficiency in bringing about the desired end result (that of attaining successful mood-stabilizing treatment), GAP-43 induction may or may not be either. There is little evidence to suggest that increased GAP-43 is necessary for the successful amelioration of bipolar symptoms, for although VPA elicited GAP-43 induction, lithium, the prototypic mood-stabilizing drug, had no effect on GAP-43 expression in our *in vitro* cell model. Nonetheless, this does not preclude the possibility that lithium may exert such an effect on GAP-43 in other cell model systems or in brain tissue. Whether GAP-43 alterations alone are sufficient for optimal bipolar therapy is another question entirely, which these data alone cannot fully address. Many agents may lead to an increase in expression of GAP-43, if by virtue of nothing other than altering cell growth and differentiation rates, but it does not necessarily follow, based on what is yet known, that all of these agents would serve as sufficient mood-stabilizing drugs.

Down-regulation of MARCKS protein may be more consistent with a mood-stabilization mechanism. The observation that both VPA and lithium, to the exclusion of a number of drugs used for various other psychotropic applications, elicited a significant reduction in MARCKS, suggests that alterations in MARCKS may be involved in the mechanism of action of these agents, and supports the identification of MARCKS as a potential molecular target which may or may not contribute to the therapeutic effects of drugs used in the treatment of bipolar disorder. However, the findings that

carbamazepine, another widely used mood-stabilizing drug therapy (although not FDA-approved for this application), as well as some less commonly used mood-stabilizing agents, including lamotrigine (data not shown) and verapamil, did not alter MARCKS expression, point to the possibility that either MARCKS is not integral to mood-stabilizing efficacy, or that the *in vitro* exposure conditions and model we employed were not optimal to allow the down-regulation we may have observed following chronic exposure *in vivo*. Another important question concerning the effects observed is that of sufficiency. The identification of MARCKS as a potential target for the action of drugs used in the treatment of bipolar disorder suggests that the down-regulation of MARCKS alone, by any method, would be enough to yield successful amelioration of bipolar symptoms. However, this diminishes the potential role of other mechanisms, such as the alterations in PI signaling induced by lithium, or the effects of VPA on GAP-43 expression. It is conceivable that the alterations in MARCKS expression observed *in vitro* would lead, *in vivo*, to further downstream events important for altering plasticity or other parameters involved in changing the course of the disorder. In other words, MARCKS may be just one target of many which may ultimately result in the efficacy of this group of drugs, those which exhibit anti-manic and/or mood-stabilizing action. This is more compatible with the notion that other drugs may be able to positively affect the course of bipolar therapy. Whereas VPA and lithium may operate, at least in part, through their effects on MARCKS, the involvement of MARCKS in bipolar therapy is indirect, and other drugs could theoretically elicit the same end result, yet through a separate pathway, perhaps bypassing MARCKS altogether.

It is interesting that VPA led to opposite effects on two CNS proteins which are both involved in cellular plasticity and intracellular signaling, and both regulated by PKC. This is despite the fact that the VPA-induced alterations of these proteins occurred over a very similar time course and dose range. These data support recent *in vivo* findings in our laboratory which imply an apparent inverse regulation of MARCKS and GAP-43 in

certain highly plastic brain regions, such as the hippocampus, in that levels of GAP-43 are high in some regions while MARCKS levels are low, and vice versa (McNamara and Lenox, 1997). This would suggest that at certain developmental stages or in particular brain regions, MARCKS and GAP-43 play alternative or opposing roles, even though they can be regulated similarly. Such comparable pharmacodynamic properties suggest that both proteins play important roles in the long-term therapeutic action of VPA.

CHAPTER 4
ROLE OF PKC IN MECHANISM OF ACTION OF VPA

Introduction

PKC is an 80-kD protein expressed at high concentrations in neuronal tissues, and the observation that PKC is the major enzyme responsible for regulation of both GAP-43 and MARCKS make the effects of VPA on PKC an interesting area of investigation. PKC is a serine/threonine kinase which, by virtue of its effects on intracellular signal transduction, is thought to play a role in hormone and neurotransmitter secretion and regulation of cell proliferation and differentiation, through mechanisms which are still not fully understood (Fujise *et al.*, 1994; Hofmann, 1997). PKC is actually comprised of a large family of at least 11 mammalian isoforms, classified into three groups based on their activation profiles. The conventional (cPKC) group includes α, βI/βII, and γ, which are regulated by Ca^{++}, diacylglycerols (DAG), negatively charged phospholipids, and phorbol esters (potent tumor promoters which mimic DAG). The novel (nPKC) group contains δ, ε, η (L), and θ, and is characterized by activation requirements similar to those of the cPKC's, but lack of Ca^{++} activation. The atypical (aPKC) members are ζ, ι (λ), and μ(D), and are all independent of Ca^{++} and DAG or phorbols, requiring only phospholipids for activation (Fujise *et al.*, 1994; Hofmann, 1997). The PKC isozymes differ in their primary structure and genomic origin, so that they are not derived from a single mRNA, but rather from a series of structurally related mRNAs coding for a family of enzymes with very similar properties. In humans, the genes for the various isozymes are located on different

chromosomes, with the exception of the βI and βII isoforms, which are the result of differential splicing (Stabel and Parker, 1991).

The primary structure of PKC contains four major regions, termed C_1 through C_4, which exhibit a high degree of homology between isotypes. These conserved regions are generally thought to represent areas involved with functions common to all PKC isozymes, such as the kinase domain. Interspersed between the conserved regions are variable regions V_1 through V_5, which exhibit a lower degree of homology and probably allow for properties specific to individual isoforms. The higher level structure of PKC is comprised of catalytic and regulatory domains. The catalytic domain alone is referred to as PKM, and has a molecular weight of 45-55 kDa. It is located in the C-terminal region of the polypeptide, and confers its kinase activity. The regulatory region, located at the N-terminal and with an approximate molecular weight of 32-36 kDa, contains phospholipid-and phorbol-binding domains responsible for modulating alterations in the activity of the enzyme. It is at the regulatory region that diacylglycerol (DAG) and phorbol esters bind to transiently activate the enzyme, eventually resulting in an increased rate of degradation (down-regulation). All members of the PKC superfamily carry in the N-terminal region at least one zinc-finger structure (more commonly two), which is thought to aid in protein-protein interactions. The N-terminal region also contains a pseudosubstrate sequence, conferring the enzyme's autoinhibitory activity. This region serves to regulate activity of the enzyme, and removal of this region results in constitutive and activator-independent kinase activity. The pseudosubstrate domain resembles the consensus phosphorylation sequence but contains no phosphorylatable residue; it controls kinase activity by occupying the catalytic site and thereby blocking access of the substrate (Stabel and Parker, 1991).

cPKC activation is dependent upon two major events: (1) formation of the ternary enzyme+Ca^{2+}+phospholipid complex (the presence of the phospholipid further enhancing the affinity of the enzyme for calcium), and (2) binding of DAG (or phorbol), leading to

the conformational changes which result in activation. Down-regulation of the enzyme follows prolonged activation, and is accomplished through both increased proteolysis and sequestration (association of the enzyme with cytoskeletal or nuclear components). Autophosphorylation of the enzyme is not a requirement, but probably plays a role (Stabel and Parker, 1991).

Data from our laboratory indicate that PKC activity is decreased over time following chronic lithium exposure, as is expression of PKC isozymes α and ε (Watson and Lenox, 1997). These findings are in agreement with those reported by Manji and colleagues, which reflected reductions in PKC activity and expression of α and ε isoforms following either lithium or VPA exposure, in either rats or C6 glioma cells (Manji *et al.*, 1993; Chen *et al.*, 1994; Manji *et al.*, 1996). Tissue distribution of PKC isozyme expression varies, and the relative expression of each isoform may contribute to its unique role in signal transduction. Individual PKC isozymes, upon activation, are differentially compartmentalized, suggesting that they mediate distinct cellular functions. PKC-γ appears to be the only isoform unique to brain and spinal cord, with no expression elsewhere; its precise role remains unknown. The same is true for most of the other isoforms - although there has been no dearth of studies into roles of the various PKC isozymes, results have been inconclusive and in some cases contradictory (to be discussed further in Discussion). Whereas PKC-α and ε have been implicated in tumor growth and suppression of apoptosis, δ may induce opposite effects (Hofmann, 1997). Both PKC-α and -δ are thought to be important in early brain development (Blackshear *et al.*, 1996), while PKC-ζ may play a role in proliferation, and several isoforms have reportedly been implicated in neurite outgrowth and/or cell differentiation (Ways *et al.*, 1994; Hundle *et al.*, 1995; Borgatti *et al.*, 1996). We have previously established that the immortalized hippocampal cell line HN33 expresses PKC isoforms α, δ, ε, and ζ, and our laboratory has demonstrated *in vitro* effects of lithium on PKC activity as well as on expression levels of various PKC isozymes (Watson and Lenox, 1997). In light of previous reports

demonstrating VPA-induced alterations in PKC in a non-neuronal cell line, it will be of interest to investigate the effects of VPA exposure on these parameters in HN33 cells, especially given preliminary evidence from our laboratory for effects of VPA on MARCKS and GAP-43 in these cells. It has been suggested that the colocalization of some isoforms with substrate(s) may ensure preferential and rapid phosphorylation following PKC activation. Based on evidence indicating a down-regulation of MARCKS and increased expression of GAP-43 following chronic VPA exposure, it is predicted that VPA will activate PKC, at least transiently, so that the downstream effects on MARCKS and GAP-43 can occur as observed. If VPA does, indeed, activate PKC, both its activity level and isoform expression should decrease over time, in the characteristic pattern of down-regulation following long-term activation. This acute activation followed by chronic down-regulation has been demonstrated previously in the HN33 cells with lithium, phorbol esters, and retinoic acid.

These and other studies suggest that VPA has significant effects on both PKC and its substrates, though the connection between such effects is unclear. Although one would expect PKC alterations to be in large part responsible for any further downstream effects on its substrates MARCKS and GAP-43, this direct cause-and-effect relationship has yet to be established. The following studies will therefore help to establish whether or not PKC is necessary to the downstream effects on MARCKS and GAP-43, and which isoforms may be most important for each effect. After first establishing the ability of VPA to effect alterations in both PKC activity and expression of various PKC isozymes in HN33 cells, the next objective will be to address the role of PKC in altering MARCKS and GAP-43 levels. VPA-induced alterations in PKC-specific activity will be measured indirectly by phosphorylation of the substrate MBP, and associated alterations in PKC isoform expression will be measured using Western immunoblotting techniques. PKC inhibitors will then be used to assess whether PKC activity is necessary to elicit the observed VPA-induced alterations in MARCKS and GAP-43, and isoform-specific

antisense oligonucleotides will be used to narrow down which isoforms are important for each parameter. It is hypothesized that PKC activity will be necessary to any observed effects by VPA on MARCKS or GAP-43, and that inhibition of the enzyme will block such observed effects.

Results

Effect of VPA Exposure on PKC Activity in HN33 Cells

HN33 cells exposed to 0.6 mM VPA for 15 min - 3 d were assayed for effects on PKC activity, as measured by MBP phosphorylation (Figure 4.1). Acute VPA exposure (15 min - 4 h) resulted in a tendency toward increased membrane-associated activity and a correlated decrease in PKC activity in the cytosolic fraction. This paradigm most closely follows a translocation phenomenon, whereby cytosolic PKC is shuttled into the membrane for activation. By the 3 d time point, activity in the membrane fractions is down-regulated ($p<0.05$). Similar results were observed using MARCKS peptide as the phosphorylation substrate (data not shown).

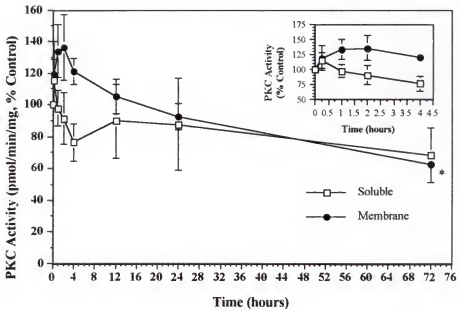


Figure 4.1. Effect of VPA exposure on PKC activity in HN33 cells. VPA exposure leads to apparent PKC activation, followed by down-regulation. HN33 cells exposed to 0.6 mM VPA were processed and soluble and membrane fractions isolated as detailed in Materials and Methods (Chapter 2). Assay was performed utilizing the PKC activators PMA (phorbol), $CaCl_2$, and phosphatidylserine. PKC activation was measured by the γ-^{32}P phosphorylation (in pmol/min/mg) of the PKC substrate MBP. Mean activity of control samples was 102 ± 8.1 pmol/min/mg. The figure shows the overall pattern of PKC activity from 15 min (acute) to 3 d (chronic) VPA exposure; the inset shows PKC activation only at acute time points (up to 4 h). Results are mean $\pm$ S.E.M. of at least four determinations, and are expressed as percent of activity in untreated control samples. *$p<0.05$

Effect of VPA on PKC-α

PKC-α is observed entirely in the soluble fraction of HN33 cells. As shown in Figure 4.2, exposure to 0.6 mM VPA results in an acute (15 min – 12 h) increase in PKC-α expression, followed by down-regulation over time ($p<0.05$).

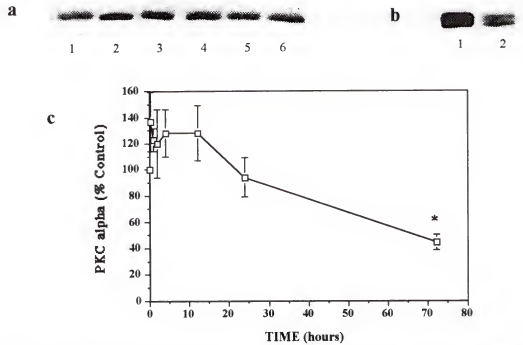


Figure 4.2. Effect of VPA on PKC-α expression in soluble fraction of HN33 cells. (a) Representative Western immunoblot of soluble PKC-α expression following acute (24 h or less) VPA exposure @ 0.6 mM. Protein samples were loaded into lanes as follows: Lane 1, untreated control; Lane 2, 15 min VPA; Lane 3, 1 h VPA; Lane 4, 4 h VPA; Lane 5, 12 h VPA; Lane 6, 24 h VPA. (b) Representative Western immunoblot of cytosolic PKC-α expression following chronic (3 d) VPA exposure @ 0.6 mM. Lane 1 was loaded with protein from an untreated control, and Lane 2 was loaded with protein from a sample exposed to VPA for 3 d. (c) HN33 cells were exposed to 0.6 mM VPA for 15 min - 3 d, and PKC-α levels (present only in the cytosolic fraction of these cells) were assessed by Western immunoblot analysis. Results are expressed as percent of untreated control, and are the mean ± S.E.M. of four determinations. *$p<0.05$

Effect of VPA on PKC-δ

PKC-δ undergoes a significant increase in expression levels in both soluble ($p<0.001$) and membrane ($p<0.0001$) fractions of cells during chronic VPA exposure (1 & 3 d), and as late as 3 d, there appears to be no down-regulation (Figure 4.3).

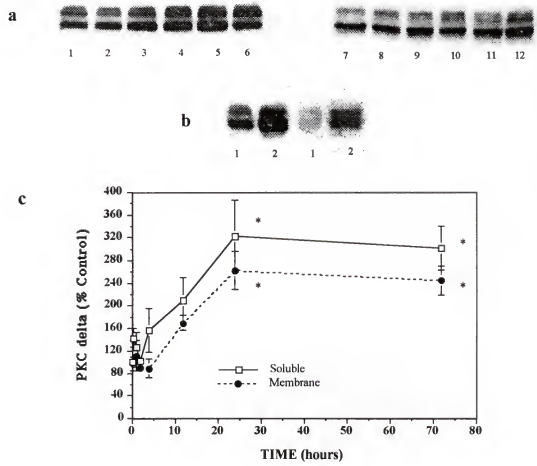


Figure 4.3. Effect of VPA on PKC-δ expression in HN33 cells. (a) Representative Western blot of PKC-δ expression following acute (<24 h) VPA exposure @ 0.6 mM. The blot at left (Lanes 1-6) shows cytosolic PKC in HN33 cells, while the blot at right (Lanes 7-12) shows membrane-associated PKC. Protein samples were loaded into lanes as follows: Lane 1, untreated control; Lane 2, 15 min VPA; Lane 3, 1 h VPA; Lane 4, 4 h VPA; Lane 5, 12 h VPA; Lane 6, 24 h VPA; Lane 7, untreated control; Lane 8, 15 min VPA; Lane 9, 1 h VPA; Lane 10, 2 h VPA; Lane 11, 4 h VPA; Lane 12, 12 h VPA. (b) Representative Western of PKC-δ expression following chronic (3 d) VPA exposure @ 0.6 mM. The blot at left shows cytosolic PKC in HN33 cells, while the blot at right shows membrane-associated PKC. Lane 1 was loaded with protein from an untreated control, and Lane 2 was loaded with protein from a sample exposed to VPA for 3 d. (c) HN33 cells were exposed to 0.6 mM VPA for 15 min - 3 d, and PKC-δ levels were assessed by Western immunoblot analysis. Results are expressed as percent of untreated control, and are the mean ± S.E.M. of four determinations. *$p<0.001$, soluble and *$p<0.0001$, membrane.

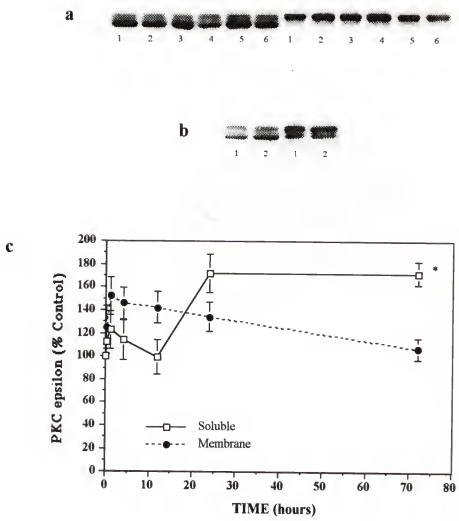


Figure 4.4. Effect of VPA on PKC-ε expression in HN33 cells. (a) Representative Western immunoblot of PKC-ε expression following acute (24 h or less) VPA exposure @ 0.6 mM. The six lanes at left show cytosolic (or soluble) PKC in HN33 cells, while the six lanes at right show membrane-associated PKC. Protein samples were loaded into lanes as follows: Lane 1, untreated control; Lane 2, 15 min VPA; Lane 3, 1 h VPA; Lane 4, 2 h VPA; Lane 5, 4 h VPA; Lane 6, 12 h VPA. (b) Representative Western immunoblot of PKC-ε expression following chronic (3 d) VPA exposure @ 0.6 mM. The blot at left shows cytosolic (or soluble) PKC in HN33 cells, while the blot at right shows membrane-associated PKC. Lane 1 was loaded with protein from an untreated control, and Lane 2 was loaded with protein from a sample exposed to VPA for 3 d. (c) Cells were exposed to 0.6 mM VPA for 15 min - 3 d, and PKC-ε levels were assessed by Western immunoblot analysis. Results are expressed as percent of untreated control, and are the mean ± S.E.M. of four determinations. *$p<0.05$

Effect of VPA on PKC-ε

As shown in Figure 4.4, membrane-associated PKC-ε increases acutely (15 min - 4 h), and then returns to basal levels by the 3 d time point. Cytosolic PKC-ε expression remains close to baseline acutely, and then increases significantly following prolonged (1-3 d) VPA exposure ($p<0.05$). This pattern of expression reflects an apparent activation and translocation of PKC-ε, whereby PKC is activated, shuttled from cytosol into the membrane (where the enzyme is active), and then down-regulated and returned from membrane to cytosol following prolonged activation.

Effect of VPA on PKC-ζ

PKC-ζ shows little change in expression at early time points, and then is up-regulated sometime between 1 d and 3 ($p<0.0001$), with a similar tendency toward up-regulation observed in the soluble fraction (Figure 4.5).

Effect of PKC-α Antisense Oligonucleotides on PKC-α Expression in HN33 Cells

Antisense oligonucleotides were designed to bind to various segments of the mouse and/or human PKC-α mRNA sequence, and block its translation to protein. Figure 4.6 and Table 4.1 list the antisense oligonucleotides and show the regions of mouse PKC-α gene they were designed to bind. All oligonucleotides were synthesized with phosphorothioate backbones, in order to increase their stability in culture. A series of experiments was then conducted to down-regulate PKC-α expression in HN33 cells, first using a single antisense oligonucleotide sequence, then using the combination of five antisense oligonucleotides. A sense oligonucleotide was used as the negative control for these experiments.

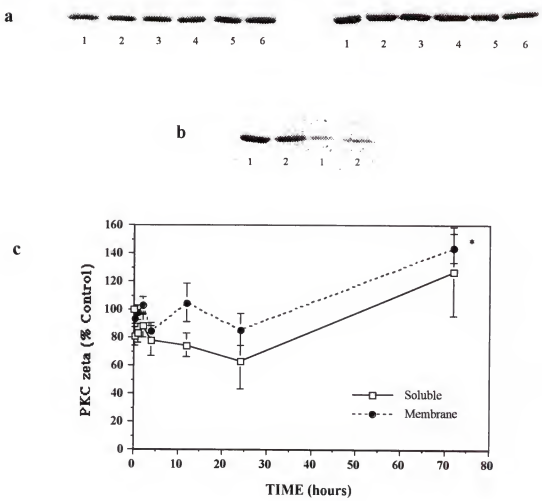


Figure 4.5. Effect of VPA on PKC-ζ expression in HN33 cells. (a) Representative Western immunoblots of PKC-ζ expression following acute (24 h or less) VPA exposure @ 0.6 mM. The blot at left shows cytosolic (or soluble) PKC in HN33 cells, while the blot at right shows membrane-associated PKC. Protein samples were loaded into lanes as follows: Lane 1, untreated control; Lane 2, 15 min VPA; Lane 3, 1 h VPA; Lane 4, 4 h VPA; Lane 5, 12 h VPA; Lane 6, 24 h VPA. (b) Representative Western immunoblot of PKC-ζ expression following chronic (3 d) VPA exposure @ 0.6 mM. The blot at left shows cytosolic (or soluble) PKC in HN33 cells, while the blot at right shows membrane-associated PKC. Lane 1 was loaded with protein from an untreated control, and Lane 2 was loaded with protein from a sample exposed to VPA for 3 d. (c) HN33 cells were exposed to 0.6 mM VPA for 15 min - 3 d, and PKC-ζ levels were assessed by Western immunoblot analysis. Results are expressed as percent of untreated control, and are the mean ± S.E.M. of four determinations. *$p<0.0001$, membrane

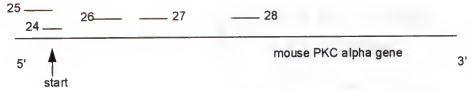


Figure 4.6. PKC-α antisense oligo-nucleotide design. This figure depicts the antisense oligonucleotides designed to bind to PKC-α mRNA and block its translation. Shown is the representative PKC-α gene from mouse (as reported in the NCBI database), and the five oligonucleotides (24, 25, 26, 27, 28) designed to bind to the nucleotide sequence at the indicated, base-complementary regions. Start codon is demarcated by the arrow; both oligonucleotides 24 and 25 span this region. Not shown is #23, the sense oligonucleotide designed as a negative control, which reads as the sense complement to antisense oligo 24. All oligonucleotides are 20-base phosphorothioate linked, with the exception of #23 and #24, which are 15-base phosphorothioates, and all were cross-referenced through GenBank to ensure unique sequence identity with PKC-α.

Table 4.1 lists the 5' to 3'nucleotide sequences of each PKC-α oligonucleotide (ON) by number, and the species specificity of each.

Table 4.1. Antisense oligonucleotide sequences.

ON #	Sequence of Nucleotides	Species Specificity
23	ACCATGGCTGACGTT	mouse & human
24	AACGTCAGCCATGGT	mouse & human
25	CAGCCATGGTTCCCCCCAAC	mouse
26	AGAAGGTAGGGCTTCCGTAT	mouse & human
27	GTTACCAACAGGACCTAGCT	mouse
28	TGAGTTCAGCATGACATTGT	mouse

Initial experiments attempted to establish the optimal dose range for oligonucleotide exposure and protein down-regulation. Oligonucleotide concentrations ranging from 0.1 to 1.0 μM (the standard concentrations reported in similar studies) had no effect on cell viability or PKC-α expression following 24 h exposure (data not shown). Increasing oligonucleotide concentration to 10 μM and lengthening exposure time to 48 h had no effect on PKC-α expression (data not shown).

Next, methods were employed to increase oligonucleotide uptake and compensate for potential oligonucleotide degradation. In keeping with previous reports of successful antisense-mediated protein down-regulation, the cationic carrier lipid LipofectAMINE (Gibco BRL, Gaithersburg, MD) was used at 5 μg/ml and 15 μg/ml, and cells were serum-deprived during oligonucleotide exposure. In addition, medium and its constituents were replaced fresh every 12 h during exposure. PKC-α expression remained at basal levels following 24 or 48 h exposure to oligonucleotide under these conditions (data not shown).

The final approach utilized phorbol to down-regulate PKC expression (non-selectively), then PKC-α antisense oligonucleotides were added following phorbol removal, in an effort to selectively block PKC-α recovery. A representative Western is shown in Figure 4.7. HN33 cells were exposed to 1.0 μM phorbol 12,13-dibutyrate (PDBu) for 24 h, after which cells were washed with media to remove drug. Next, cells were exposed to 1.0 μM sense or antisense oligonucleotides (total of five antisense oligonucleotides) directed to PKC-α (Figure 4.6). Cells were exposed to oligonucleotides for a total of 24 h, but antisense-treated cells showed no difference in PKC-α expression than sense-treated or untreated control cells. As shown in Figure 4.7, there is clearly no difference in PKC-α expression between those PDBu-pre-treated cells which are antisense-treated or untreated. PDBu exposure resulted in a reversible down-regulation of PKC-α (Lane 2 vs. 1), and PKC-α antisense failed to prevent the recovery of PKC-α expression at rates comparable to control levels (Lanes 7&8 vs. 3-6). Whereas toxicity commonly arises as a problem in antisense applications in cell and tissue culture, the HN33

cells showed no evidence of cytotoxicity following 24-48 h exposure to any of the pharmacological agents at any of the concentrations tested.

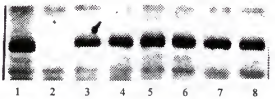


Figure 4.7. Effect of PKC-α antisense oligonucleotides on PKC-α expression in HN33 cells. Shown is a representative Western immunoblot of PKC-α expression in HN33 cells following antisense treatment. Lane 1 holds an untreated control; Lane 2 shows PKC-α expression in cells treated with 1.0 μM PDBu for 24 h; Lane 3 shows cells treated with 1.0 μM PDBu for 24 h, then no treatment for 24 h; Lane 4 shows cells treated with 1.0 μM PDBu for 24 h, then LipofectAMINE for 24 h; Lane 5 shows cells treated with 1.0 μM PDBu for 24 h, then sense oligo #23 for 24 h; Lane 6 shows cells treated with 1.0 μM PDBu for 24 h, then sense oligo #23 and LipofectAMINE for 24 h; Lane 7 shows cells treated with 1.0 μM PDBu for 24 h, then antisense oligonucleotides #24, 25, 26, 27, and 28, each at 1.0 μM, for 24 h; Lane 8 shows cells treated with 1.0 μM PDBu for 24 h, then antisense oligonucleotides #24, 25, 26, 27, and 28, plus LipofectAMINE, for 24 h.

Effect of PKC Inhibitor LY333531 on VPA-Induced Down-Regulation of MARCKS

LY333531 alone had no effect on MARCKS expression, but when combined with VPA, LY333531 prevented the VPA-induced MARCKS down-regulation observed in the membrane, but not soluble, fraction of HN33 cells (Figure 4.8). MARCKS expression in both cytosol and membrane of VPA-treated cells was significantly different from that of LY333531-treated cells ($p<0.005$, soluble, $p<0.0001$, membrane). Cytosolic MARCKS in combined VPA/LY333531-treated cells was significantly different from that of LY333531-treated cells ($p<0.005$), indicating that, even in the presence of the PKC inhibitor LY333531, VPA was able to induce down-regulation of cytosolic (but not membrane-associated) MARCKS expression. In contrast, LY333531 prevented the VPA-induced down-regulation of MARCKS in the membrane fraction of HN33 cells.

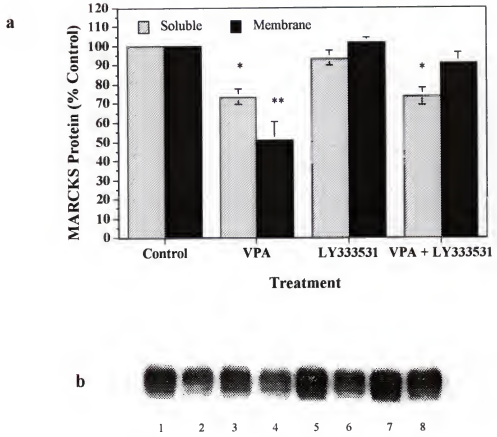


Figure 4.8. Effect of PKC inhibitor LY333531 on VPA-induced down-regulation of MARCKS. (a) HN33 cells were exposed for 24 h to 1.0 mM VPA with or without 1.0 μM LY333531. MARCKS expression was quantitated by Western blot analysis, and results (expressed as percent of untreated control) reflect the mean ± S.E.M. of at least five determinations. *$p<0.005$, soluble; *$p<0.0001$, membrane (b) Representative Western blot showing MARCKS expression in the presence of VPA ± LY333531. Lanes 1-4 represent cytosolic MARCKS, and were treated for 24 h as follows: control, untreated (1); 1.0 mM VPA (2); 1.0 μM LY333531 (3); 1.0 mM VPA + 1.0 μM LY333531 (4). Lanes 5-8 show membrane-associated MARCKS, and were treated for 24 h as described for 1-4 (respectively) above.

Effect of PKC Inhibitor LY333531 on VPA-Induced GAP-43 Induction

LY333531, alone or in combination with VPA, had no effect on GAP-43 expression in HN33 cells (Figure 4.9). GAP-43 levels in both soluble and membrane fractions are significantly increased ($p<0.05$) in both VPA-treated groups, compared to

controls (untreated or LY333531-treated). Therefore, the PKC inhibitor LY333531 was unable to prevent the VPA-induced GAP-43 increase, for even in the presence of LY333531, VPA increased GAP-43 expression in both soluble and membrane fractions. These data indicate that PKC inhibition alone is insufficient to prevent the GAP-43 increase observed in HN33 cells following 24 h 1.0 mM VPA exposure.

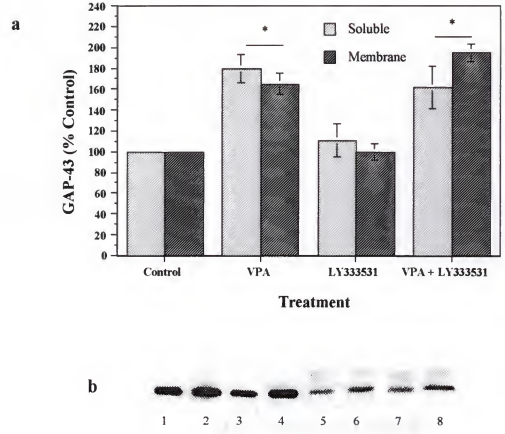


Figure 4.9. Effect of PKC inhibitor LY333531 on VPA-induced GAP-43 increase. (a) HN33 cells were exposed for 24 h to 1.0 mM VPA with or without 1.0 μM LY333531. GAP-43 expression was quantitated by Western blot analysis, and results (expressed as percent of untreated control) reflect the mean ± S.E.M. of three determinations. *$p<0.05$ (b) Representative Western blot showing GAP-43 expression in the presence of VPA ± LY333531. Lanes 1-4 represent membrane-associated GAP-43, and were treated for 24 h as follows: control, untreated (1); 1.0 mM VPA (2); 1.0 μM LY333531 (3); 1.0 mM VPA + 1.0 μM LY333531 (4). Lanes 5-8 show cytosolic GAP-43, and were treated for 24 h as described for 1-4 (respectively) above.

Discussion

Inasmuch as PKC activation has been associated with regulation of expression of both GAP-43 and MARCKS, the effects of VPA on PKC isozyme levels and PKC activity were investigated more directly, in an effort to better assess the role of PKC in the mechanism of action of VPA in the brain. Whereas lithium, the first-line anti-manic/mood-stabilizer, is believed to operate in part through the PI signaling pathway, our group and others have provided evidence against such a mechanism for VPA (Vadnal and Parthasarathy, 1995; Lenox *et al.*, 1996; Dixon and Hokin, 1997; Watson *et al.*, 1998), although both drugs have similar effects on MARCKS expression.

Our findings indicate that, like lithium, VPA induces alterations in expression of PKC isoforms α and ε (Watson and Lenox, 1997). However, the time course for VPA-induced changes is earlier than that for lithium, and PKC-ε expression actually increases (acutely in membrane, chronically in cytosol), in contrast to the reduction previously reported for both lithium and VPA (Chen *et al.*, 1994; Manji *et al.*, 1996; Watson and Lenox, 1997). Chen *et al.* (1994) reported that chronic VPA exposure of C6 glioma cells reduced the expression of PKC isozymes α and ε in intact cells, a finding similar to that observed following chronic lithium administration in the same cell model (Manji *et al.*, 1993). Subsequent studies revealed PKC-induced alterations in multiple components of the β-adrenergic receptor-coupled cAMP-generating system, including β_1-AR, $G\alpha_s$, and adenylyl cyclase, each of which is phosphorylated by PKC (Chen *et al.*, 1996). The significance of these changes is unclear, but together they suggest a major role for PKC in the VPA- or lithium-induced molecular effects underlying their efficacy, and may be instructive when other PKC-related effects are compared between the two drugs.

In this study, VPA exposure resulted in an apparent transient activation of PKC (within 2 h), followed by a gradual reduction in activity with chronic exposure (4 h and beyond). PKC activity was significantly down-regulated by the 72 h time point. Similar

results were reported following exposure of HN33 cells to the phorbol PMA, though activation occurred within 15 minutes and down-regulation of PKC activity was evident by 1 h (Watson *et al.*, 1994). This finding is further in agreement with previous reports which showed that chronic VPA or lithium exposure of C6 glioma cells significantly reduced PKC activity in both membrane and soluble fractions (Chen *et al.*, 1994; Manji *et al.*, 1996). The observed patterns of alterations in protein activity and expression suggest that isoforms α and ε might, under these conditions, be involved in the phosphorylation and regulation of MARCKS protein expression, since both VPA and lithium elicited alterations in PKC-α and -ε expression in HN33 cells, and the time course of MARCKS alterations roughly corresponds to that of the changes observed in PKC isoform expression. Indeed, previous studies have indicated the ability of these two isoforms to phosphorylate MARCKS, whereas PKC-ζ is unable to phosphorylate MARCKS *in vitro* (Fujise *et al.*, 1994; Herget *et al.*, 1995; Uberall *et al.*, 1997). One could hypothesize a process in which VPA, directly or indirectly, activates PKC, especially isoforms α and/or ε, at early time points (between 0 and 12 h). By 3 d, there is a significant down-regulation of activity in the membrane and a comparable drop in the cytosol. Consistent with this hypothesis are data which show an increase in PKC activity within 15 seconds following phorbol exposure (Stabel and Parker, 1991), and a time course for MARCKS down-regulation which closely follows the time course of PKC activation in HN33 cells exposed to phorbol (Watson *et al.*, 1994).

While VPA and lithium both affect PKC isozyme α (and perhaps ε), the two drugs have very different effects on PKC-δ. VPA induces a chronic up-regulation in PKC-δ, whereas lithium has little or no effect on PKC-δ expression (Watson and Lenox, 1997). In comparing VPA and lithium, one might hypothesize that such differences in patterns of PKC activation underlie the differences observed in downstream effects such as protein expression or cell growth and morphology. For example, the up-regulation of PKC-δ resulting uniquely from VPA exposure is probably not important for the MARCKS down-

regulation observed, but might be involved in the GAP-43 induction observed following VPA but not lithium exposure; in support of this hypothesis is evidence indicating that PKC-δ and -ε phosphorylate GAP-43 (Oehrlein *et al.*, 1996). Indeed, in our study, the GAP-43 increase occurred over a time course and of a magnitude similar to those of the PKC-δ alterations observed. Conversely, PKC isozymes α and/or ε may play a role in the observed VPA- and lithium-induced down-regulation of MARCKS.

Reports are in disagreement as to the role of PKC in neuronal differentiation. A number of studies have suggested that, rather than being a positive regulator of the process, PKC may actually be an inhibitory influence, so that suppression of the enzyme results in increased process outgrowth. For example, use of various PKC inhibitors, and subsequent reductions in PKC activity, have been associated with increased neurite outgrowth (Heikkila *et al.*, 1989; Parodi *et al.*, 1990; Tsuneishi, 1992; Wooten, 1992; Jalava *et al.*, 1993; Ekinci and Shea, 1997). Long-term down-regulation of PKC activity with phorbols such as PMA has provided similar results (Tsuneishi, 1992; Carlson *et al.*, 1993; Jalava *et al.*, 1993; Ekinci and Shea, 1997), with one study showing enhanced neurite outgrowth following combined phorbol and PKC inhibitor treatment (Heikkila *et al.*, 1989). However, some of these same studies have further shown that down-regulation of PKC activity alone is insufficient to elicit a differentiated phenotype (Ekinci and Shea, 1997; Carlson *et al.*, 1993; Wooten, 1992).

On the other hand, numerous reports have presented equally convincing evidence for a positive role for PKC in regulating neuronal differentiation and a positive role in maintaining the functionally active state (Coleman and Wooten, 1994; Cabell and Audesirk, 1993; Parrow *et al.*, 1992; Abraham *et al.*, 1991; Tonini *et al.*, 1991), though this role is probably not unique to PKC. Previous data have implicated a number of different PKC isoforms in cellular differentiation, including all four of the isotypes present in HN33 cells: α, δ, ε, and ζ (Wada *et al.*, 1989; Leli *et al.*, 1992, 1993; Ways *et al.*, 1994; Hundle *et al.*, 1995; Borgatti *et al.*, 1996). Indeed, the subcellular localization and

activation of PKC isozymes are regulated in an isoform-specific manner during neurogenesis, suggesting they are involved in control of neural development and in neuronal differentiation (Oehrlein *et al.*, 1998).

The role of PKC in proliferation and differentiation remains unclear, for reports differ as to which PKC isoform(s) is/are responsible for various aspects of cellular differentiation. Some studies have attributed to PKC-α a role as the intermediate in cellular differentiation, as a result of alterations in PKC-α mRNA and/or protein expression observed concomitant with differentiation of PC12 and other cell lines (Borgatti *et al.*, 1996; Parrow *et al.*, 1995; Murray *et al.*, 1993; Leli *et al.*, 1993; Tonini *et al.*, 1991; Wada *et al.*, 1989). However, these reports differ in their mechanistic conclusions, for in three studies, PKC-α expression increased with differentiation (Borgatti *et al.*, 1996; Parrow *et al.*, 1995; Murray *et al.*, 1993), while in three other studies, PKC-α and -ε levels had decreased following comparable treatment and neuritogenesis (Leli *et al.*, 1993; Tonini *et al.*, 1991; Wada *et al.*, 1989). Leli's group further showed that the intracellular delivery of PKC-α and -ε isoform-specific antibodies resulted in a morphologically differentiated phenotype in SH-SY5Y cells (Leli *et al.*, 1992).

While some groups observed no differentiation-associated alterations in isoforms other than PKC-α, a number of reports have indicated that in various cell lines, other members of the PKC superfamily are involved. Borgatti's group, who showed that PKC-α was up-regulated during PC12 cell differentiation, found in the same study that PKC δ, ε, and ζ were down-regulated (Borgatti *et al.*, 1996). A number of studies have provided evidence for a role of PKC-ε in regulating neuronal differentiation, since neurite outgrowth has been associated with altered PKC-ε levels (Ponzoni *et al.*, 1993; Parrow *et al.*, 1995; Fagerstrom *et al.*, 1996; Ekinci and Shea, 1997; Hundle *et al.*, 1997; Zeidman *et al.*, 1999). In support of the notion that PKC-ε may be involved in the process, as suggested by Leli's and Wada's work (Leli *et al.*, 1993; Wada *et al.*, 1989), a separate

study showed that overexpression of PKC-ε but not PKC-δ led to an increase in NGF-induced neurite outgrowth in stably transfected PC12 cell lines (Hundle *et al.*, 1995). In stark contrast to these findings, yet another group reported just the opposite in NIH 3T3 cells, in that PKC-δ overexpression induced a morphological change and reduced growth rate consistent with differentiation that was not observed in cells overexpressing PKC-ε (Mischak *et al.*, 1993). Further, PKC-ζ has been similarly examined in transfected leukemic cells, and results indicate that PKC-ζ overexpression is able to induce cellular differentiation (Ways *et al.*, 1994). Another study that utilized antisense techniques also emphasized the importance of PKC-ζ in NGF-induced neuronal differentiation (Coleman and Wooten, 1994).

All of these studies point to different PKC isozymes as mediators of similar effects. These apparently contrasting results may merely be a function of the varying experimental methods and conditions used from one study to the next, such as different cell lines, exposure conditions, and other parameters. These findings may alternatively indicate that (1) each isozyme plays its own, specialized role in the complex process of cellular differentiation, and (2) alterations in the level of each isozyme may be compensated for by alterations in the levels and actions of other isozymes. Further studies will need to be conducted in order to answer these questions, to confirm more definitively the association between each PKC isoform and the parameter in question, but these data strongly suggest that both VPA and lithium may down-regulate MARCKS via a PKC-dependent mechanism. On the other hand, the unique role of the PI signaling pathway in the action of lithium, and the differential pattern of activation of the PKC isozymes by VPA and lithium may indicate that there are multiple pathways to the down-regulation of MARCKS expression, and may also confer different clinical therapeutic properties on these two drugs. The findings further suggest that the action of VPA on MARCKS protein expression could be cell and/or tissue specific, for it may be modulated, in part, by the expression pattern of the PKC isozymes specific to the cell types. Indeed, Stabel and

Parker (1991) suggest that the distribution of MARCKS closely follows the distribution of PKC, based on early studies of PKC activity showing wide disparity across regions (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981).

Having established an apparent causal relationship between VPA and PKC, the purpose of the next experiments was to establish more precisely the necessity of PKC in eliciting the obesrved VPA-induced effects on MARCKS and GAP-43. In order to better establish the role of various PKC isoforms in the MARCKS down-regulation and GAP-43 increase observed, experiments were designed employing antisense technology. Through this method, antisense oligonucleotides are designed and directed to specifically bind and "knock down" the nucleic acid precursors of each individual PKC isozyme, resulting in transient elimination of the protein in question. There are four main mechanisms by which antisense oligonucleotides are hypothesized to work: (1) by inhibiting the necessary processing and transport of RNA, (2) by interfering with binding of the ribosome to the nucleotide sequence, so that translation is not initiated, (3) by inhibiting the completion of translation after the process has begun, and (4) by binding to the sequence, thereby characterizing it as abnormal and resulting in degradation by RNase H (Schlingensiepen *et al.*, 1997).

For this study, a number of trials were performed in an effort to down-regulate PKC-α in the HN33 cells. One sense and five antisense oligonucleotides were prepared by Gemini Biotech, Ltd. (The Woodlands, TX) through the DNA Synthesis Core of the UF Interdisciplinary Center for Biotechnology Research (ICBR). Each oligonucleotide was designed to bear unique identity to the PKC-α genome of either mouse or human, as reported in the National Center for Biotechnology Information database, and screened through GenBank. Various regions of the cDNA sequence were targeted, including the start codon (see Figure 4.6). In an effort to maximize stability and binding of oligonucleotides, all were composed of 15 or 20 bases each, and had phosphorothioate linkages in place of the endogenous phosphodiester backbone of DNA. Whereas toxicity

commonly arises as a problem in antisense applications in cell and tissue culture, the HN33 cells showed no evidence of cytotoxicity following 1 to 2 d exposure to 1 and 10 μM concentrations of PKC-α oligonucleotides. In initial experiments, cells were exposed to oligonucleotide(s) for 24-48 h, in an effort to establish a protocol for the consistent reduction of PKC-α levels prior to VPA exposure. When numerous efforts failed to elicit any reduction in PKC-α expression, PKC was first down-regulated through 24 h, 1 μM PDBu (phorbol) administration prior to exposure of cell cultures to oligonucleotide. Following thorough phorbol washout, test cells were exposed to all five oligonucleotides concomitantly for 24 h. In order to increase uptake of oligonucleotides, cells were deprived of serum nutrients and exposed to LipofectAMINE, an agent which serves as a cationic carrier lipid to transport oligonucleotide into the cells. Additionally, media and oligonucleotides were replaced after 12 h to compensate for any degradation which had taken place. Despite these measures designed to optimize antisense-directed repression of PKC-α levels following initial down-regulation by phorbol, the protein recovered in antisense-treated cells at about the same rate as that of drug-free controls (see Figure 4.7). Common explanations for the difficulty in achieving significant decreases in protein expression include instability of the antisense oligonucleotide sequence, limited uptake of the oligonucleotide, suboptimal binding of oligonucleotide to its target sequence, and half-life of the particular protein. Despite efforts to account for and overcome these issues, attempts to carry out further studies based on the antisense work were ultimately abandoned, due to the persistent failure of PKC-α antisense to down-regulate PKC-α expression. Perhaps, in this study, the unpredictable tertiary conformation of the antisense oligonucleotides was such that access and/or binding to the target mRNA sequence was prevented, or perhaps the oligonucleotides were sequestered and/or degraded before they even reached their destination. Because antisense technology is still in its developmental stages, and oligonucleotide design and exposure conditions vary greatly among systems,

significant time and expense may often be invested before down-regulation of the protein in question is optimized.

Whereas expression of each PKC isoform could not be down-regulated individually, overall PKC activity inhibition was achieved through the use of the PKC inhibitor LY333531 (Eli Lilly and Company, Indianapolis, IN), a direct competitor for ATP binding (Jirousek *et al.*, 1996). LY333531 is a PKC-β-selective inhibitor (at low nm concentrations) which also inhibits PKC isozymes α, δ, and ε at concentrations in the low μM range (Ishii *et al.*, 1996; Jirousek *et al.*, 1996). Exposure of HN33 cells to 1.0 μM LY333531 for 24 h resulted in no change in either MARCKS or GAP-43 expression, nor any evidence of toxicity. When combined with 1.0 mM VPA, LY333531 effectively prevented the VPA-induced MARCKS down-regulation in the membrane fraction of HN33 cells, but with no accompanying effect on the VPA-induced GAP-43 increase.

From these studies it is clear that PKC activation plays an integral role in the VPA-induced MARCKS effects observed, as inhibition of PKC-directed phosphorylation of MARCKS resulted in the inability of VPA to elicit down-regulation of membrane-associated MARCKS. It is interesting that this LY333531-induced prevention of MARCKS down-regulation by VPA occurred in membrane, the fraction of HN33 cells in which VPA exerts its greatest effect. This finding is further consistent with the known localization of PKC activity in the membrane, and also with a previous report showing that phosphorylation of MARCKS is necessary for its dissociation and translocation from membrane (Allen and Aderem, 1995). Whether PKC activation alone is sufficient to reduce MARCKS has previously been investigated by our laboratory. It was shown that phorbol esters, potent activators of PKC, yield a significant down-regulation of MARCKS in the HN33 cells, and that the time course of phorbol-induced MARCKS alterations follows closely behind the time course of phorbol-induced PKC activation and down-regulation (Watson *et al.*, 1994). These findings support the notion that activation (and perhaps also subsequent down-regulation) of PKC, in and of itself, is sufficient to elicit a

reduction in MARCKS, though PKC activation may not be the only route through which the MARCKS alterations may occur. Indeed, Brooks *et al.* (1992) reported evidence of regulation of MARCKS through both PKC-dependent and independent pathways, with platelet-derived growth factor (PDGF)-induced down-regulation of MARCKS through a post-transcriptional mechanism.

As for GAP-43, PKC activation is probably not necessary to elicit the alterations in GAP-43 observed. This is evidenced by the fact that PKC inhibition failed to block the VPA-induced increase in GAP-43 in the HN33 cells. Even with the strong GAP-43 expression observed in the membrane of these cells, and the significant up-regulation of this protein to several times basal levels, LY333531 caused no attenuation of the VPA-induced GAP-43 increase observed. Assuming conditions were optimal for significant PKC inhibition, we can conclude that PKC activation is not entirely responsible for the VPA-induced GAP-43 increase observed, nor is PKC activation alone likely to be sufficient for the effect, since lithium-induced alterations in PKC activity and isoform expression did not lead to any change in GAP-43 expression.

The findings do little to address the issue of the involvement of PKC in bipolar disorder and successful mood-stabilizing therapy. However, one can hypothesize that if MARCKS dysregulation is integral to bipolar disorder, and PKC is the primary modulator of MARCKS, then perhaps the enzyme is perturbed in some patients exhibiting symptoms of the disorder. This would be especially applicable to cases in which the patient demonstrates normal levels of MARCKS, for MARCKS, being downstream from PKC, is regulated by PKC, and if PKC parameters are abnormal, then PKC-induced MARCKS regulation will likely also be abnormal. Even if MARCKS itself is not involved in the manifestation of the disorder, PKC may be a major player. Indeed, previous studies have provided evidence for alterations in PKC-related signaling in patients with bipolar disorder. Reports have shown that in both platelets and post-mortem brain samples of individuals diagnosed with the disease, PKC translocation and activity levels were

significantly increased as compared to controls, as measured by phorbol- or serotonin-stimulated histone phosphorylation (Friedman *et al.*, 1993; Wang and Friedman, 1996). In addition, PKC isoform expression was altered in comparison to controls, with higher levels of PKC-α, δ, and ζ and lower PKC-ε expression in bipolar patients (Wang and Friedman, 1996). Further, two-week lithium treatment of subjects resulted in a reduction in both PKC translocation and activity in platelets (Friedman *et al.*, 1993). In a more recent clinical study, the PKC-selective inhibitor tamoxifen (an estrogen receptor antagonist widely used in the treatment of breast cancer) has been shown to be effective in treating acute mania in a small sample of patients (Manji *et al.*, 1999). Inasmuch as PKC is believed to play a pivotal role in regulating neuronal signal transduction and in modulating intracellular cross-talk between neurotransmitter systems (Nishizuka, 1992), these studies are suggestive of the potential for affective (mood) dysfunction as a result of alterations or dysfunction in PKC and associated neuronal signaling pathways. A definitive association between PKC alteration and mood stabilization may facilitate the development of a whole new line of agents useful in the treatment of bipolar disorder.

CHAPTER 5
EFFECT OF VPA ON CELL VIABILITY, GROWTH AND MORPHOLOGY

Introduction

While VPA has proven very useful in its clinical applications to seizures and bipolar disorders, the detrimental effects of this drug on the developing embryo have been well documented (Wiger *et al.*, 1988; Gofflot *et al.*, 1996; Menegola *et al.*, 1996). High frequencies of neural tube defects (NTDs) have been linked to prenatal VPA exposure (Bjerkedal *et al.*, 1982; Robert and Guibaud, 1982; Lindhout and Schmidt, 1986; Wiger *et al.*, 1988; Gofflot *et al.*, 1996; Menegola *et al.*, 1996). Such defects may include open neural tube, spina bifida, anencephaly, meningomyelocele, and encephalocele, and are thought to be caused by a number of different factors, such as environmental or pharmacological teratogens, genetic anomalies, and various maternal predisposing factors (Rhoads and Mills, 1986; Norman *et al.*, 1995). In more recent years, folic acid and/or methionine supplementation have been shown to reduce the frequency of NTDs, both in the general population and in offspring of mothers undergoing anticonvulsant therapy (Laurence *et al.*, 1981; Smithells *et al.*, 1981; Rhoads and Mills, 1986; Trotz *et al.*, 1987; Mulinare *et al.*, 1988; Nosel and Klein, 1992; Ehlers *et al.*, 1996). Therefore, women are advised to discontinue VPA therapy or take folic acid supplements during and just before pregnancy. The mechanism of this protective effect is not understood. Folate is known to be a precursor of methionine, an important amino acid for cell proliferation, and vitamin B12 is an essential cofactor for methionine synthesis. In mothers of NTD-affected offspring, circulating serum folate levels are low but not clinically deficient; the same is

true for B12 levels (Laurence *et al.*, 1981; Rhoads and Mills, 1986; Kirke *et al.*, 1993). In addition, homocysteine, the direct precursor for methionine, is found at high levels in the serum of some mothers in this population (Steegers-Theunissen *et al.*, 1994; Mills *et al.*, 1995, 1996). This evidence suggests that, in at least a fraction of NTD cases, the mothers of NTD-afflicted offspring exhibit a metabolic insufficiency, perhaps at the level of the homocysteine-to-folate conversion step. This "metabolic defect" hypothesis is in contrast to the simpler notion of a nutritional deficiency (of either folate or methionine), and is consistent with the possibility that VPA therapy is one factor which may induce or enhance this metabolic defect. It is of note that, in experimental studies, *in vivo* supplementation of either nutrient to pregnant animals resulted in decreased frequencies of NTDs in offspring, while *in vitro* studies did not reproduce the protective effect (Trotz *et al.*, 1987; Hansen and Grafton, 1991; Nosel and Klein, 1992; Hansen *et al.*, 1995; Ehlers *et al.*, 1996). Preliminary studies in our laboratory further support the notion that the effect is of a complicated metabolic nature, for folate or methionine supplementation to cell growth medium did not prevent the VPA-induced down-regulation of MARCKS observed in HN33 cells (Watterson unpublished data).

In light of the deleterious developmental effects of this drug, the effects of VPA on cell growth and differentiation merit in-depth investigation. Previous studies, while few in number, have provided evidence for a dose-dependent VPA-induced inhibition of cell proliferation via arrest at mid-G1 of the cell cycle (Martin and Regan, 1991), as well as VPA-induced differentiation of neuroblastoma and glioma cells (Regan, 1985; Cinatl *et al.*, 1996). The inhibition of cell proliferation, alterations in cellular interactions, and altered differentiation have all been correlated with prenatal drug teratogenicity, especially of the developing neural tube (Wilk *et al.*, 1980; Mummery *et al.*, 1984; Martin *et al.*, 1988). It is plausible that arrest of cell proliferation at a specific phase in the cell cycle, accompanied by irreversible differentiation, may result in the effects observed to be consistent with prenatal teratogenicity.

The experiments of this chapter have been designed to address further the issues of cell growth and morphology, and the effects VPA has on these parameters. The purpose of the following experiments is to ascertain whether VPA induces alterations associated with differentiation in the same cell model in which altered MARCKS and GAP-43 expression were observed. Based on the significantly increased GAP-43 levels discussed previously (in Chapter 4), it is hypothesized that VPA will increase neurite outgrowth, since both processes (increased GAP-43 and outgrowth of neurites) generally accompany cell differentiation, as described in depth in Chapter 3. Alterations in cell growth rate and/or cellular morphology would be consistent with the hypothesis that VPA is capable of modifying cytoplasticity. This is in contrast to previous observations showing a lack of effect by lithium on HN33 cell growth or morphology. It has already been demonstrated, through these studies and others, that these two drugs operate through different mechanisms, and have different profiles clinically and at a molecular level. In addition, previous reports reflect VPA-induced modifications of cell growth and differentiation state (Martin and Regan, 1991; Regan, 1985). If VPA were indeed found to elicit alterations in cell growth and morphology, the next step would be to address the mechanism of these effects by examining the role of PKC. As discussed in Chapter 4, PKC-specific inhibitors (LY333531 and bisindolylmaleimide, or BIM, Calbiochem, La Jolla, CA) will be used in an attempt to block the VPA effect, to test the hypothesis that PKC activity is necessary for the morphological changes.

In addition to the HN33 cells, PC12 cells will be employed to assess the potential of VPA to serve as a differentiating and neuroprotective agent. PC12 cells are a well-characterized cell line widely used for studying various aspects of cellular differentiation. PC12 cells exhibit NGF sensitivity while HN33 cells do not, a characteristic which will be brought to bear upon these studies. In this apoptotic cell death model, NGF-differentiated PC12 cells will be used to assess the ability of VPA to protect against cell death and/or neurite retraction induced by removal of NGF.

These experiments will further address the question of the effects of VPA on cell plasticity. The ability of VPA to alter cell plasticity is relevant to its unique pharmacological profile, which includes both anticonvulsant and long-term mood-stabilizing effects in the adult brain, as well as teratogenic effects on the developing nervous system. All of these major effects would seem to suggest altered plasticity (either therapeutic or abnormal) as a contributing factor.

Results

Effect of VPA on HN33 Cell Division

HN33 cells were exposed to 0.6 mM VPA for up to 7 d. At this therapeutically relevant concentration, an inhibition of cell growth was observed, evident in the increased cell doubling time ($p<0.01$; Table 5.1). Despite this effect on cell number, no effect on cell viability was observed when cells were plated at high confluence, even at a higher concentration of VPA (1.0 mM), as shown in Table 5.2 (no significance). This supports the notion that the reduction in cell number is a result of decreased growth rate and not due to cell death, since it was only observed under conditions in which cell division was possible.

Table 5.1 shows the mean (± S.E.M.) time required for the HN33 cell population to double in number when exposed to either no drug (control) or chronic 0.6 mM VPA. *$p<0.05$ (t-test)

Table 5.1. Cell Doubling Time	
Control	19 ± 0.1 h
0.6 mM VPA	26 ± 1.4 h *

Table 5.2 shows the mean (± S.E.M.) number of cells per viewing field in cultures plated too densely to allow for neurite outgrowth. No significant effect was observed.

Table 5.2. # Cells / Field	
Control	2,970 ± 280
1.0 mM VPA	3,583 ± 103

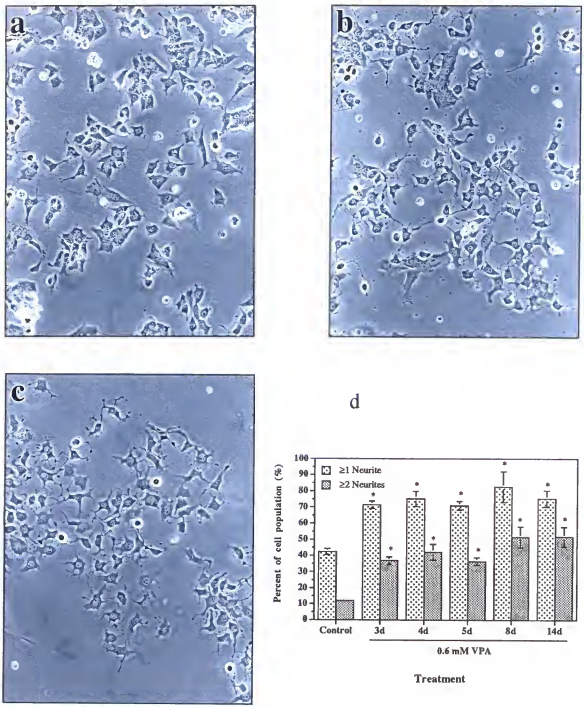


Figure 5.1. VPA-induced neurite outgrowth in HN33 cells. These photomicrographs show (a) untreated HN33 cells; (b) HN33 cells exposed to 0.6 mM VPA for 3 d; and (c) HN33 cells exposed to 0.6 mM VPA for 14 d. The figure (d) summarizes quantitatively the effect of VPA on HN33 cell morphology. Cells were exposed to 0.6 mM VPA for varied lengths of time, and then cells were photographed and analyzed. Data are presented as mean percent (± S.E.M.) of cells with one neurite or more, and mean percent (± S.E.M.) of cells with two neurites or more; a neurite was defined as having length equaling at least the diameter of the respective cell body. *$p<0.0001$

Effect of VPA on HN33 Cell Morphology

In comparison to control cells grown in parallel in the absence of drug, an increase in neurite outgrowth was observed in VPA-exposed cells as early as 3 d in culture, and persisted for the duration of the exposure (Figure 5.1). The percentage of cells with one neurite or more increased significantly ($p<0.0001$) between 0 and 3 d, and remained at about 71-76% throughout the duration of VPA exposure, from 3 to 14 d. The percentage of cells with two neurites or more also increased ($p<0.0001$) between 0 and 3 d (from 11.7% in controls to 36.6% in VPA-treated cells), and increased again ($p<0.0001$) between 5 and 8 d (to 52%). It was also noted, during cell collection and harvesting, that HN33 cells exposed to VPA concentrations higher than 0.3 mM for at least 1 d exhibited an increased adherence to culture flasks, as evidenced by the greater effort required to remove cells by sloughing with versene (data not shown). This greater adherence to substrate is consistent with the increased outgrowth of processes and increased expression of GAP-43, and together are suggestive of differentiation to a more mature state. It was also observed that cell density significantly influenced neurite outgrowth of HN33 cells. Even in the absence of drug treatment, there was an inverse correlation between number of cells in the viewing field and percent of these cells exhibiting neurites ($p<0.0005$; data not shown).

Effects of the PKC Inhibitor LY333531 on VPA-Induced Alterations in HN33 Cell Number and Neurite Outgrowth

HN33 cells were exposed to 1.0 mM VPA and/or 1.0 μM LY333531 for 48 h. As depicted in Figure 5.2a, cell number was decreased significantly only in cultures treated with the combination of VPA and LY333531 ($p<0.05$), though cultures treated with either drug alone exhibited a tendency toward decreased growth. Figure 5.2b shows the significant effect of either VPA or LY333531 on neurite outgrowth in HN33 cells. Either agent increased neurite outgrowth as compared to controls ($p<0.01$), and the two agents

combined yielded a greater effect than either agent alone ($p<0.05$). These results suggest that PKC exerts a negative influence on cell differentiation, so that addition of a PKC inhibitor allows for increased neurite outgrowth. Further, the mechanism of VPA-induced increase in neurites is perhaps not activation of PKC, but either long-term down-regulation of PKC, or some alternate mechansim altogether. The observations that VPA and LY333531 exhibited additive effects on both neurite outgrowth and cell number are suggestive of different mechanisms.

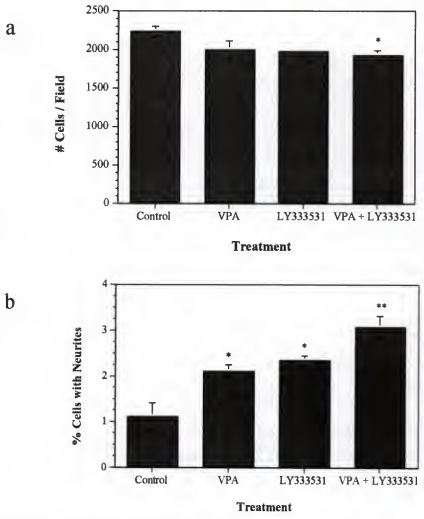


Figure 5.2. Effects of VPA and LY333531 on HN33 cell density and neurite outgrowth. HN33 cells were exposed to either 1.0 mM VPA or 1.0 mM LY333531, or the combination, for 48 h. Cells were then photographed and analyzed to determine (a) cell number and (b) percent of cell population with neurites. Data are presented as (a) mean number of cells in the viewing field (± S.E.M.; *$p<0.05$) and (b) mean percent of cell population with neurites (± S.E.M.; *$p<0.01$; **$p<0.05$).

Effect of VPA on Neurite Outgrowth in PC12 Cells

Similar to the effects of VPA observed in HN33 cells, a concentration-dependent increase in neurite outgrowth in PC12 cells occurred following 2 d exposure to therapeutic concentrations of VPA ($p<0.05$), as shown in Figure 5.3. In cells exposed to a higher concentration of VPA (1.8 mM), neurite outgrowth appeared to be no different from that of controls. It is of note that, similar to effects observed in HN33 cells, the PC12 cells exhibited an inverse correlation between cell density and neurite outgrowth ($p<0.005$; data not shown).

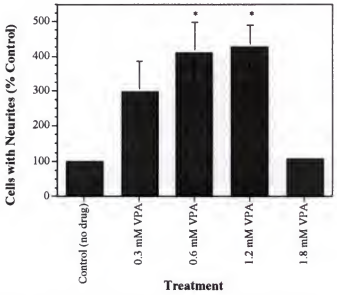


Figure 5.3. VPA-induced neurite outgrowth in PC12 cells. Cells were exposed to VPA for 2 d, and then photographs were taken for comparison. Data are presented as mean percent (± S.E.M.) of cells displaying one neurite or more. *$p<0.05$

Effects of VPA and the PKC Inhibitor BIM on PC12 Cell Number and Neurite Outgrowth

Non-differentiated PC12 cells exposed to either 0.6 mM or 1.8 mM VPA exhibited no alteration in growth rate compared to controls, as evidenced by lack of difference in cell density (Figure 5.4 a and b). Similarly, 200 nM BIM, either alone or in combination with VPA, elicited no alteration in overall cell density. Following exposure to 0.6 mM

VPA, PC12 cells exhibited increased neurite outgrowth, which was unaffected by the addition of 200 nM BIM ($p<0.05$; Figure 5.4c). Neither BIM nor 1.8 mM VPA, alone or in combination, had any effect on neurite outgrowth in PC12 cells (Figure 5.4d). These results suggest that, at concentrations of VPA therapeutic for mood-stabilization (e.g., 0.6 mM), PC12 cells exhibit increased neurite outgrowth, and that this effect is independent of PKC.

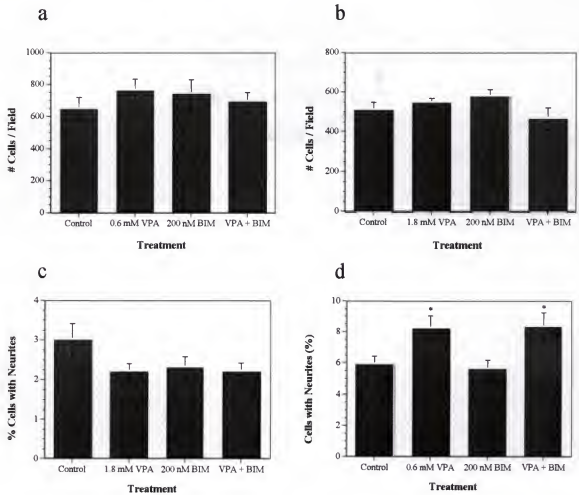


Figure 5.4. Effects of VPA and BIM, a PKC Inhibitor, on PC12 Cell Density and Neurite Outgrowth. Non-differentiated PC12 cells were exposed to 0.6 mM VPA, 1.8 mM VPA, 200 nM BIM, or some combination thereof for 24-48 h. Shown in (a) and (b) are mean cell density (± S.E.M.) following exposure to 0.6 mM VPA and 1.8 mM VPA, respectively. Shown in (c) and (d) are percent of total population exhibiting neurites (± S.E.M.) following exposure to 0.6 mM VPA, 1.8 mM VPA, 200 nM BIM, or a combination. *$p<0.05$

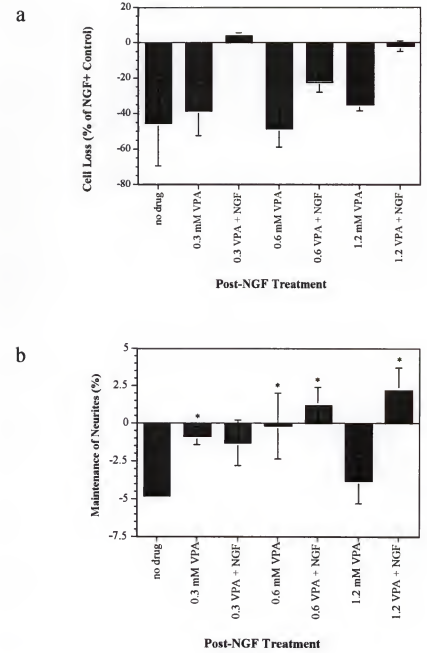


Figure 5.5. Effect of VPA on NGF-Differentiated PC12 Cells. Differentiation of PC12 cells was induced by 4-7 d exposure to 100 ng/ml NGF, and then NGF was either removed or replaced, and VPA was added at various concentrations (0.3, 0.6, 1.2 mM). NGF-positive control is represented as baseline (0 on y-axis). (a) Cell Density. Shown is mean (± S.E.M.) number of cells, expressed as a percent of NGF-positive control values. No protective effect was observed. (b) Neurite Outgrowth. Shown is mean percent (± S.E.M.) of cell population exhibiting neurites in treatment groups compared to NGF-positive controls. Protection against loss of neurites was observed. *$p<0.05$

Neuroprotective Effect of VPA on NGF-Differentiated PC12 Cells

Following NGF-induced differentiation of PC12 cells, varying concentrations of VPA (0.3, 0.6, 1.2 mM) were added, alone or in combination with NGF (100 ng/ml). NGF-deprived cultures experienced a loss of neurites and decreased cell number (attributable to cell death), as shown in Figure 5.5. VPA-treated cultures (alone or in combination with NGF) exhibited a decreased rate of neurite loss, though this effect was no greater than that elicited by NGF alone ($p<0.05$; Figure 5.5b). Exposure to the combination of VPA and NGF resulted in a tendency, albeit insignificant, toward decreased cell death, while VPA alone had no apparent effect (Figure 5.5a). These results suggest that VPA is protective against neurite loss in NGF-differentiated PC12 cells, but not protective against cell loss associated with trophic factor deprivation.

Discussion

HN33 Cells

Exposure of HN33 cells to therapeutic concentrations (up to 1.0 mM) of VPA for up to 14 d produced no apparent cytotoxicity, and cells appeared healthy and viable when assessed by Trypan blue dye exclusion assay (data not shown). However, a concentration-dependent increase in cell doubling time was noted. Following long-term exposure, cellular growth rates were notably slower for HN33 cells exposed to 0.6 mM VPA. This effect was reversible upon removal of the drug, and could be avoided altogether by plating cells more densely (Table 5.2). These findings are similar to reported effects of VPA on glioma (C6) and neuroblastoma (neuro 2A, UKF-NB-2, UKF-NB-3) cells, in which VPA exhibited a reversible anti-proliferative effect which was not attributable to cytotoxicity

(Martin and Regan, 1991; Cinatl *et al.*, 1996). In one particular study, VPA was found to arrest cells at the post-mitotic mid-G1 phase of the cell cycle (Martin and Regan, 1991).

The next parameter assessed in the HN33 cells was that of differentiation. Following chronic (>1 d) exposure to therapeutic concentrations of VPA, cells exhibited a significant increase in neurite outgrowth consistent with previous reports (Martin and Regan, 1991; Cinatl *et al.*, 1996). The increase in neurite outgrowth observed in this study occurred over a time course similar to that of the MARCKS down-regulation observed (see Chapter 3). While no significant alterations in either parameter were observed at 1 d following 0.6 mM VPA exposure, both cell morphology (neurite outgrowth) and MARCKS expression had undergone significant changes by 3 d. Following continued VPA exposure, little or no change was evident in either MARCKS or cell morphology between 3 d and 5 or 6 d, but a significant increase in neurite outgrowth was observed by the 8 d time point, which corresponds roughly to the further decrease in MARCKS protein observed between 6 and 10 d (see Figure 3.5).

Although these similarities in overall time course are interesting, they may simply point to the notion that VPA-induced alterations in both cell morphology and MARCKS expression are effects observed only following chronic, but not acute, exposure to this drug. Previous studies would suggest that these two parameters are unrelated, for in this particular cell model, chronic lithium exposure also resulted in significant reductions in MARCKS expression, but with no accompanying change in cell morphology (Watson and Lenox, 1996). Further, exposure of HN33 cells to either of the VPA metabolites hydroxyvalproic acid or 2-propylglutaric acid, whose structures are analagous to that of VPA, produced no change in cell morphology, and only one metabolite, at high concentrations, had a notable effect on MARCKS.

On the other hand, retinoic acid, a known differentiating agent, has been shown to elicit both MARCKS down-regulation and neurite outgrowth in these cells (Watson *et al.*, 1994). Further, the effect of lithium on MARCKS differs from that of VPA in the HN33

cells. Whereas lithium appears to exert its greatest effect on MARCKS in the soluble fraction of these cells, and requires a longer exposure, VPA exposure results in a greater reduction in MARCKS expression, at a much earlier time point (3 d for VPA vs. 10 d for lithium), and the effect is most pronounced in the membrane fraction of these cells. As has been suggested previously, VPA and lithium likely operate through two different or only partially overlapping mechanisms. While lithium works in part through its effect on the PI signaling system, VPA does not. Similarly, VPA exposure leads to effects on cell growth and morphology which may or may not be related to its therapeutic efficacy. Indeed, the observed VPA-induced effects more than likely contribute to this drug's unique therapeutic profile, as will be further discussed in Chapter 6.

It is clear from previous studies that morphological alterations are not a necessary prerequisite for down-regulation of MARCKS protein expression, for lithium exposure results in MARCKS alterations without accompanying changes in HN33 cell growth or morphology. Whether MARCKS down-regulation is necessary for the kinds of changes in cell growth and morphology necessary to cell differentiation is a more difficult question to answer based on the data presented, but will be further discussed below. Future studies might further address this phenomenon in a variety of cell lines simply by assessing MARCKS expression levels following differentiation.

As for GAP-43 and its potential involvement in the morphological alterations observed, there is extensive evidence in the literature linking GAP-43 with neurite outgrowth, as discussed in Chapter 3. In this study, both parameters (GAP-43 and neurite outgrowth) continued to increase throughout the length of VPA exposure examined. This, along with the fact that lithium had no effect on either GAP-43 expression or neurite outgrowth (data not shown), suggests that the effects are somehow related. Although necessity and/or sufficiency have not been addressed sufficiently by the data presented, there is a wealth of evidence which supports the notion that increased GAP-43 levels greatly contribute to the overall process of neurite outgrowth and cell differentiation. As

discussed in Chapter 3, neurite outgrowth is accompanied by a large increase in GAP-43 expression, and is thought to help stabilize growth cone morphology and promote growth cone adhesion (Aigner and Caroni, 1995). Antisense-directed depletion of GAP-43 in growth cones results in the absence of spreading, branching, and adhesion of growth cones (and therefore, neurite outgrowth) in primary sensory neurons (Aigner and Caroni, 1993). Suppression of GAP-43 expression has been found to yield adverse effects on axon outgrowth both *in vivo* and *in vitro* (Meiri *et al.*, 1998; Benowitz and Routtenberg, 1997; Strittmatter *et al.*, 1995; Jap Tjoen San *et al.*, 1992; Shea *et al.*, 1991), and overexpression of GAP-43 induced nerve sprouting in the adult nervous system of transgenic mice (Aigner *et al.*, 1995). Even in non-neuronal cells, expression of a GAP-43 transgene has been found to induce extensive process outgrowth accompanied by reorganization of the membrane cytoskeleton (Strittmatter *et al.*, 1994; Yankner *et al.*, 1990; Zuber *et al.*, 1989). Skene and Willard (1989) have suggested that the induction of GAP (GAP-43 and other growth-associated proteins) expression is necessary to post-injury remodeling, and that the inability of many areas of the CNS to induce high levels of GAP expression may in part underlie the failure of CNS axons to regenerate after axonal injury, whereas areas of the peripheral nervous system retain the ability to up-regulate GAP-43 as well as to regenerate axons after injury. The presence of GAP-43 is probably not necessary to neurite outgrowth *per se*, but rather, plays an important role in regulating precisely the various parameters involved in the overall event, such as navigation or pathfinding (Aigner and Caroni, 1995). According to this "GAP hypothesis", certain growth associated proteins, such as GAP-43, provide a mechanism for regulating neurite outgrowth and structural plasticity in the nervous system (Skene and Willard, 1989).

With regard to PKC activity and isoform expression, and their association with the VPA-induced neurite outgrowth observed in HN33 cells, all effects occurred at the same therapeutically relevant concentration of VPA (0.6 mM). The alterations in PKC activity appear to precede the morphological effects, for membrane-associated PKC activation,

followed by down-regulation, occurred within hours following addition of VPA to the culture medium, whereas neurite outgrowth was observed only following chronic VPA exposure. Similarly, alterations in PKC isoform expression were observed acutely, within 1-2 h following VPA addition, as well as following longer-term VPA exposure (1-3 d).

Although reports are inconclusive and even contradictory as to the either positive (Abraham *et al.*, 1991; Parrow *et al.*, 1992; Cabel and Audesirk, 1993; Coleman and Wooten, 1994) or negative (Heikkila *et al.*, 1989; Parodi *et al.*, 1990; Tsuneishi, 1992; Carlson *et al.*, 1993; Jalava *et al.*, 1993; Chakravarthy *et al.*, 1995; Ekinci and Shea, 1997) regulatory role of PKC in neuronal differentiation, each of the four PKC isozymes present in HN33 cells has, in one report or another, been implicated in neurite outgrowth and/or cellular differentiation, as discussed at length in Chapter 4. The present study supports the association of neurite outgrowth with either one or any combination of the following: (a) acute induction followed by down-regulation of PKC-α expression, (b) chronic up-regulation of PKC-δ, (c) apparent translocation (consistent with activation) of PKC-ε from cytosol to membrane, followed by down-regulation, and/or (d) chronic up-regulation of PKC-ζ. The findings discussed herein are consistent with previous studies showing alterations in PKC-α mRNA and/or protein expression observed concomitant with differentiation of PC12 and other cell lines (Borgatti *et al.*, 1996; Parrow *et al.*, 1995; Murray *et al.*, 1993; Leli *et al.*, 1993; Tonini *et al.*, 1991; Wada *et al.*, 1989). However, these reports differ in their mechanistic conclusions, for in three studies, PKC-α expression increased with differentiation (Borgatti *et al.*, 1996; Parrow *et al.*, 1995; Murray *et al.*, 1993), while in three other studies, PKC-α levels had decreased following comparable treatment and neuritogenesis (Leli *et al.*, 1993; Tonini *et al.*, 1991; Wada *et al.*, 1989). The findings presented herein are also consistent with reports which have provided evidence for a role of PKC-ε in regulating neuronal differentiation, since neurite outgrowth has been associated with altered PKC-ε levels (Ponzoni *et al.*, 1993; Parrow *et al.*, 1995; Fagerstrom *et al.*, 1996; Ekinci and Shea, 1997; Hundle *et al.*, 1997; Zeidman *et*

al., 1999). Leli and Wada observed increased expression of PKC-ε concomitant with neurite outgrowth (Leli *et al.*, 1993; Wada *et al.*, 1989), and another report indicated that overexpression of PKC-ε led to an increase in NGF-induced neurite outgrowth in stably transfected PC12 cell lines (Hundle *et al.*, 1995). Still other reports emphasized the importance of PKC-ζ in neuronal differentiation, showing that PKC-ζ overexpression is able to induce cellular differentiation in transfected leukemic cells (Ways *et al.*, 1994), and antisense-mediated down-regulation of PKC-ζ inhibits NGF-induced neurite outgrowth in PC12 cells (Coleman and Wooten, 1994).

In this study, inhibition of PKC by LY333531 alone increased neurite outgrowth in HN33 cells compared to that of untreated controls. LY333531 also enhanced the VPA-induced increase in neurite outgrowth, and decreased HN33 cell density, when the two drugs were combined. These data suggest that, rather than serving as the necessary intermediate for the effects of VPA on HN33 cell growth and morphology, as initially hypothesized, one or more isoforms of PKC may actually be inhibitory toward cell differentiation. PKC inhibition not only elicited effects on its own, but enhanced the observed effects of VPA on these parameters. Such findings suggest that VPA may operate through inactivation of PKC to elicit observed effects on cell growth and morphology, or perhaps through a separate mechanism altogether. The finding that PKC was not a necessary intermediate in the VPA-induced GAP-43 induction observed (Chapter 4) is consistent with these data, since increased GAP-43 expression is associated with cell differentiation, as discussed in Chapter 3.

A novel finding observed, but not quantified, in this study was the increased adherence of VPA-treated cells to the tissue culture flasks in which they were grown. Another group reported a similar effect in C6 glioma and primary astrocytes (Martin *et al.*, 1988). Further investigation revealed that VPA induced a significant increase in the expression of the collagen type IV receptor, a heat-sensitive protein thought to be responsible for the increased adherence to substrate (Martin and Regan, 1988). The heat

shock proteins specifically respond to environmental stress, including that of teratogenic burden. This reversible, stress-induced effect is suggestive of a mechanism for the teratogenic effects of VPA.

PC12 Cells

Insofar as VPA induced morphological alterations in HN33 cells, we next investigated the effect of VPA on PC12 cells, a model in which neurite outgrowth and differentiation have been well characterized. Indeed, exposure of PC12 cells for 2 d to therapeutic concentrations of VPA yielded a significant and dose-dependent increase in neurite outgrowth. Future studies may examine alterations in PKC substrates in these cells following chronic VPA exposure, in order to strengthen the link already proposed for protein expression during such cellular events.

The PKC inhibitor BIM was used to assess the involvement of PKC in the observed VPA-induced increase in neurite outgrowth in PC12 cells. It was found that BIM alone had no effect on either PC12 cell density or neurite outgrowth. Similarly, a high concentration of VPA (1.8 mM, well above therapeutic) had no effect on either parameter. At a therapeutic concentration of VPA (0.6 mM), however, neurite outgrowth increased with or without addition of BIM, suggesting that (1) VPA, at concentrations therapeutic for mood stabilization, may serve as a differentiating agent in PC12 cells, and (2) VPA-induced differentiation of these cells does not involve PKC, as inhibition of the enzyme had neither a positive nor negative impact on the effect of VPA.

These findings are consistent with those observed following VPA exposure in the HN33 cells, insofar as therapeutic concentrations of VPA elicited increased neurite outgrowth, a process which did not appear to rely on PKC. However, the findings in PC12 cells differ from those of the HN33 cells, in that PKC inhibition did not enhance the

VPA-induced neurite outgrowth, nor did it have any effect when added alone. Further, PC12 cell growth was unaffected by either VPA or BIM, or the combination.

A number of recent studies have investigated the role of PKC in neuroprotection. Results are conflicting, but often support such a role for PKC. In a model of ischemic preconditioning-induced neuroprotection (utilizing a two-vessel occlusion protocol), altered distribution and levels of PKC-γ were observed in rat hippocampal CA1 and neocortex regions, suggesting an association between PKC and neuroprotection (Shamloo and Wieloch, 1999). Yet, in either E18 rat cortical cultures or hippocampal slices, preconditioning studies utilizing oxygen-glucose deprivation or anoxic depolarization paradigms, respectively, showed no evidence for a role of PKC, either stimulatory or inhibitory, in these mechanism of anoxic preconditioning-induced neuroprotection (Perez-Pinzon and Born, 1999; Tauseka *et al.*, 1999). Other studies have also suggested a positive role for PKC in neuroprotection. Cerebral ischemia and glutamate- or NMDA-induced cell death have been shown to follow PKC inactivation (Pizzi *et al.*, 1996; Tremblay *et al.*, 1999). Further, neuroprotective agents including low-dose amyloid precursor protein, which increases uptake of the excitatory amino acids aspartate and glutamate (Masliah *et al.*, 1998), thyrotropin-releasing hormone, which protects against NMDA toxicity (Pizzi *et al.*, 1999), and vasoactive intestinal peptide, which was found to be neuroprotective against ibotenate-induced white matter lesions (Gressens *et al.*, 1998), all were dependent upon PKC activation for their overall neuroprotective effect. In contrast, still other reports suggest that PKC activity actually contributes to neurotoxicity. In toxicity models involving anoxia, glucose deprivation, or nitric oxide toxicity, growth factors were found to protect against cytotoxicity and increase cell survival, independent of PKC (Boniece and Wagner, 1993; Maiese and Boccone, 1995). Down-regulation or inhibition of PKC alone prior to insult was found to be cytoprotective, supporting the notion that PKC is not neuroprotective, but rather, a contributor to toxicity in certain models.

One series of experiments was designed to test the hypothesis that VPA had neuroprotective activity in addition to its neurite-stabilizing role. Inasmuch as various animal models have provided evidence for progressive seizure-induced neuronal damage, VPA has been investigated as a potential neuroprotective agent (Jallon, 1997). Furthermore, lithium was shown in a recent report to be neuroprotective against NMDA-mediated glutamate-induced excitotoxicity (apoptosis) in cortical, hippocampal, and cerebellar granule cells. This effect, which apparently involved modulation of NMDA-mediated Ca^{++} influx, was both time- and concentration-dependent, and independent of inositol concentration (therefore independent of IMPase) and not a result of alterations in either NMDA receptor composition or number (Nonaka *et al.*, 1998). Other studies, in cultured cerebellar granule cells as well as rat frontal cortex, hippocampus, and striatum, showed chronic lithium-induced up-regulation of BCL-2 mRNA and protein levels, as well as protection against glutamate-induced cytotoxicity (Chen and Chuang, 1999; Manji *et al.*, 1999). Bcl-2 is a proto-oncogene thought to be protective against cell death arising from a number of different pathways, including apoptosis, and may also play a role in the regulation of neural cell differentiation (Vaux, 1993; Naumovski and Cleary, 1994; Yang and Korsmeyer, 1996; Zhang *et al.*, 1996).

NGF-differentiated PC12 cells were used as a model for neuroprotection, as NGF deprivation following cell differentiation leads to apoptotic cell death. In this study, VPA was added to NGF-differentiated cells, either alone or in combination with NGF, and both cell number and neurite outgrowth were measured. No effect of VPA on cell density was observed; VPA-treated cultures exhibited a loss in cell number similar to that observed in NGF-deprived cultures. With regard to neurite outgrowth, VPA, alone and in combination with NGF, appeared to protect differentiated PC12 cells against loss of neurites following NGF withdrawal. These findings are consistent with those observed in non-differentiated PC12 and HN33 cells, in that VPA induced neurite outgrowth and did not stimulate (at times, even inhibited) cell growth. VPA appears to have selective effects

on different cell populations. With regard to the neuroprotective potential of VPA against apoptosis, findings are inconclusive: VPA exhibited protective tendencies toward neurites, but did not protect against cell loss, following NGF-induced differentiation and subsequent withdrawal in PC12 cells. These findings agree with recent preliminary observations by Watson (personal communication), which show no evidence for neuroprotection by acute (8 h) VPA exposure against glutamate-induced cytotoxicity in hippocampally-derived HT-22 cells. The ability of anticonvulsants such as VPA to decrease the frequency or severity of epileptic episodes does not necessarily confer or reflect neuroprotective properties. Such drugs are prophylactic against further seizure-induced damage, but these drugs are not generally considered to possess the power to correct the pathology underlying epilepsy (Jallon, 1997). Instead, these studies suggest that perhaps VPA is able to stabilize existing synaptic connections. Alternatively, VPA may have protected and induced differentiation in cells which had not already differentiated in response to NGF, while cells which were NGF-differentiated were not protected by VPA and subsequently died.

Additional Comments

The ability of VPA to effect changes in plasticity correlates well with previous evidence presented by other groups showing significant developmental effects of this drug. It has been known for some time that epileptic women have a greater incidence of birth defects in their offspring than do nonepileptics, and that certain anticonvulsant drugs used to treat these women are largely responsible for this teratogenicity (Golbus, 1980; Nakane *et al.*, 1980). VPA has been the focus of many studies in this regard, as its prenatal use has been associated with a high incidence of offspring born with various dysmorphic birth defects, collectively referred to as the Fetal Valproate Syndrome (Daston *et al.*, 1995; Ardinger *et al.*, 1988; Wiger *et al.*, 1988; Winter *et al.*, 1987; Chessa and Iannetti, 1986;

Tein and MacGregor, 1985; DiLiberti *et al.*, 1984; Clay *et al.*, 1981; Nau *et al.*, 1981; Dalens *et al.*, 1980). In addition to a number of somatic defects consistently observed, infants exposed prenatally to VPA also exhibited a strikingly higher incidence of neural tube defects than the general population (Bjerkedal *et al.*, 1982; Robert and Guibaud, 1982; Lindhout and Schmidt, 1986).

A number of animal studies have shown that prenatal exposure to VPA at physiologically relevant plasma levels results in a tendency toward decreased size and weight of offspring (Ehlers *et al.*, 1996; Hansen and Grafton, 1991), as well as increased incidence of embryolethality (Hansen *et al.*, 1995b; Ehlers *et al.*, 1996). There is also overwhelming evidence for the teratogenic effects of VPA on the rodent neural tube: data indicate that even a single treatment with VPA at critical developmental times can result in such neural tube defects as exencephaly, spina bifida aperta, and spina bifida occulta in offspring (Hansen *et al.*, 1995b; Trotz *et al.*, 1987). Hansen's group showed that offspring of mice exposed *in vivo* on gd 8 to one subcutaneous injection of VPA exhibited a significant increase in the rate of exencephaly as compared to controls (Hansen *et al.*, 1995b); in a separate study, *in vitro* rat embryos exposed to VPA for 44 h beginning on gd 9 exhibited a higher incidence of open neural tubes (Hansen and Grafton, 1991). Other studies have shown similar teratogenic abnormalities, as well as defects in cranial nerve development, of *in vitro* cultured rat, mouse, or chick embryos exposed to VPA for just 24-48 h (Chatot *et al.*, 1984; Kelly and Regan, 1992; Nosel and Klein, 1992; Gofflot *et al.*, 1996). Reports reflect comparable increases in exencephaly and other neural tube defects in mouse fetuses briefly exposed to VPA *in vivo* during early gestation (Trotz *et al.*, 1987; Ehlers *et al.*, 1996). These effects are indicative of a disruption in formation of the neural tube, rather than simply a neurodevelopmental delay (Kelly and Regan, 1992).

Both retrospective and prospective studies have attempted to examine this phenomenon, but the pattern of malformations caused by VPA appears to vary greatly across species (Ehlers *et al.*, 1996), and findings differ from one report to the next. One

striking consistency among all studies is the selectivity of VPA for defects of the midline. Perhaps the specificity of action of VPA on G1 phase of the cell cycle may help explain the drug's specificity in inducing neural tube defects (Martin and Regan, 1991). Some studies suggest peak plasma levels of VPA itself, and not its metabolites, are responsible for the drug's teratogenicity; it has also been suggested that the drug accumulates to a greater degree in embryonic target tissues relative to maternal plasma concentrations (Menegola et al., 1996; Kelly and Regan, 1992; Martin and Regan, 1991). Thus far, no consensus has been reached as to the putative mechanism of this drug's teratogenic effects, and data are equally inconclusive with regard to preventive regimens. Whereas folic acid supplementation has been reported to prevent anticonvulsant-induced neural tube defects in rats and mice *in vivo* (Ehlers *et al.*, 1996; Nosel and Klein, 1992; Trotz *et al.*, 1987), the *in vitro* evidence is not as compelling. *In vitro* reports in general have shown a failure of folic acid or its metabolites to prevent VPA-induced neural tube defects in mouse or rat embryos (Hansen and Grafton, 1991; Hansen *et al.*, 1995). Additionally, preliminary findings from our laboratory, in which we used the immortalized hippocampal cell model to investigate the preventive effect of folic acid or methionine (a folic acid metabolite) on VPA-induced MARCKS down-regulation, showed no prevention or reversal of the MARCKS effect (data not shown). These data suggest that folic acid alone is not responsible for prevention of the effects elicited by VPA, but rather, that a more complex, unknown, metabolic phenomenon is somehow altered by folic acid supplementation, which in turn allows modulation of VPA-induced effects.

These findings suggest an effect of VPA on cellular differentiation during early developmental stages, and our data are consistent with previous findings. Such dramatic and selective effects on central nervous system development suggest that VPA may be altering the cellular developmental timing program at a molecular level. In accordance with this hypothesis, cells would experience a shift in their developmental timing program, from the growth phase, in which they divide and multiply, to the differentiating phase,

during which growth stops and specialized functional differentiation begins. The key is that this differentiation commences at a time prior to growth completion, and therefore before the cells are organized at a tissue level; as a result, the whole organism exhibits such gross abnormalities as open neural tube. Our findings and those of others (Martin *et al.*, 1988), in which chronic VPA exposure resulted in decreased growth rates and increased morphological differentiation, are consistent with this hypothesis. Regan's group further suggests that agents such as VPA, which significantly alter mitosis and cell-substratum adhesivity, can be predicted to induce neural tube defects, for cell proliferation and related functions are known to be integral to neural tube formation (Martin and Regan, 1988).

It is also of note that, while prenatal VPA exposure often leads to neural tube defects, lack of MARCKS protein results in similar effects. In a transgenic mouse model homozygous null for the MARCKS gene *Macs*, all pups died perinatally and exhibited a number of neural tube and other midline defects (Stumpo *et al.*, 1995; Blackshear *et al.*, 1996). Swierczynski *et al.* (1996) subsequently showed that transgenic expression of the human MARCKS gene *MACS* in these knockout mice effectively rescued the MARCKS-deficient phenotype and allowed for survival of the transgenic mice. These studies suggest that MARCKS is essential for normal CNS development, and that lack of MARCKS results in abnormal development of various midline structures, and even death. These findings are of interest in light of the present study, in which we have presented evidence for VPA-induced down-regulation of MARCKS and associated morphological alterations. Our findings suggest a putative mechanism for the developmental abnormalities associated with prenatal VPA exposure. That there is extensive evidence of VPA-induced neural tube defects, and that VPA elicits a down-regulation in MARCKS, a protein without which normal neural tube development cannot proceed, is intriguing. While our findings are merely suggestive, and more extensive studies will need to be done, this is perhaps the

first attempt to attribute MARCKS (or lack thereof) with a significant role in the mechanism of VPA-induced neural tube defects.

Beyond the developing CNS, such VPA-induced effects are likely to be relevant to plastic or regenerating areas of the adult brain, as well. Alterations in growth, morphology, and PKC substrate expression have implications for mood stabilization as well as for seizure therapy. Indeed, studies have shown that seizures induce structural and functional alterations in the brain, at cellular and molecular levels, which lead to further downstream changes in gene expression, cell morphology and function (Jallon, 1997). The notion of synaptic remodeling as the underlying means for the drug- or therapy-induced stabilization of mood disorders has also been supported by some reports, as previously discussed. One study found increased levels of N-CAM-immunoreactive proteins in the cerebrospinal fluid of a population of bipolar patients (Poltorak *et al.*, 1996), which may be suggestive of abnormal synaptic plasticity in affected individuals. Lithium and VPA affect transcriptional factors which regulate gene expression, and it is known that long-term changes in neuronal synaptic function are dependent upon the selective regulation of gene expression (Manji and Lenox, 1994). Lamotrigine was recently shown to alter neuronal hyperexcitability via inhibition of both Na^{+} and Ca^{++} channels, perhaps signifying modification of synaptic plasticity, as the authors suggest (Xie and Hagan, 1998). These findings are suggestive of changes in synaptic plasticity as a putative mechanism by which anti-bipolar therapy facilitates long-term changes in affect.

Perhaps the dramatic effects observed in this study on cellular protein expression and growth/morphology, in some combination, result in alterations in cellular signaling which are responsible for the therapeutic effects of VPA. In other words, VPA-induced molecular alterations may underlie some functional change, as yet undefined, which is responsible for modifying CNS plasticity at a cellular level. This may, in turn, allow for remodeling of the tissues in select areas of the brain, such as the limbic system, to better accommodate appropriate cell signaling events, such as those responsible for mood. As

we have shown, these molecular events occur following chronic but not acute VPA exposure, and this speaks to the therapeutic relevance of our findings: the functional changes necessary for long-term mood stabilization must be such that they lag in onset and are not easily reversed.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

VPA has proven to be very useful in its application to both seizure disorders and bipolar disorders. Because of the relative dearth of mood-stabilizing therapies available today, it is the latter application on which these research efforts have been focused. Over the past two decades, a number of reviews have been published on the efficacy of VPA in treating bipolar affective disorder (Keck and McElroy, 1996; McElroy and Keck, 1993; McElroy, 1989; Hayes, 1989; Brown, 1989; Fawcett, 1989). From various studies, it has become clear that VPA elicits a significant antimanic response rate, even in lithium-refractory patients and others who display more resistant forms of bipolar disorder, such as the rapid cycling variant (Calabrese and Delucchi, 1989; Brown, 1989). Further, VPA has proven to be quite useful and safe in combination with lithium or as a long-term prophylactic drug therapy (Granneman *et al.*, 1996; Hayes, 1989). Because of its broad therapeutic range (actually thought to be even broader than the formerly established 50-100 μg/ml range), VPA can be prescribed with less consideration of risk and less close monitoring. In fact, VPA has received more attention as a first-line mood stabilizer (before lithium or carbamazepine), because of its efficacy, relatively rapid onset of action, favorable side effect profile, and relative ease of administration (McElroy and Keck, 1993).

The findings of our current and previous studies are summarized in Table 6.1. Both chronic VPA and lithium share the property of regulating MARCKS expression, at clinically relevant concentrations (0.6 - 1.0 mM VPA was used for all experiments in this study), to the exclusion of all other psychotropic agents examined to date. Comparisons between VPA and lithium are instructive, for these two FDA-approved mood-stabilizing

agents share a striking number of differences, and further investigation may begin to answer some very important questions regarding pathophysiology as well as drug therapy. Indeed, the mood-stabilizing properties of these two drugs may be partially attributable to the similarities observed on a cellular level (such as the similar effects on PKC-α), whereas their differences, including morphological and cell growth effects of VPA, may contribute to the unique anticonvulsant properties of VPA as well as its severe developmental effects. The fact that only VPA and lithium elicit a significant reduction in MARCKS suggests that alterations in MARCKS may be involved in the mechanism of action of these agents, and supports the identification of MARCKS as a potential molecular target for the action of drugs used in the treatment of bipolar disorder. Based on previous reports, MARCKS is likely to play an important role in modulating CNS plasticity. Although the precise role of MARCKS alterations remains unknown, the identification of common targets in the brain for these drugs under conditions that reflect both effective therapeutic concentrations and the time course of clinical action may provide a unique window into the pathophysiology of bipolar disorder. It may be that MARCKS is an intermediate, and that something downstream of MARCKS is responsible for the anti-bipolar effect of these drugs. Even if the regulation of MARCKS is a clinically irrelevant "side effect" of mood-stabilizing drug therapy, it may be a useful marker nonetheless. Such findings may offer us opportunities for the design and development of the next generation of mood stabilizers, compounds which will ideally possess broad spectrum of efficacy, minimal side effects, and widened therapeutic index, all of which will contribute to increased patient compliance.

On the other hand, the finding that carbamazepine, another widely used mood-stabilizing drug therapy (although not FDA-approved for this application), as well as some less commonly used mood-stabilizing agents, including lamotrigine (data not shown) and verapamil, did not result in alterations in MARCKS expression, points to the possibility that either (1) MARCKS down-regulation itself is not integral to mood-stabilizing efficacy, or (2) the *in vitro* exposure conditions and model employed were not optimal to

allow the down-regulation which may have been observed following chronic exposure *in vivo*. Alternatively, MARCKS may activate some protein or process downstream which is responsible for the anti-manic, mood-stabilizing efficacy of these drugs; while lithium and VPA arrive at this downstream signal via MARCKS, carbamazepine and other anti-bipolar drugs may arrive at the same signal through some alternate mechanism, so that all anti-bipolar drugs do actually converge upon the same pathway ultimately, albeit through different routes.

Whereas MARCKS down-regulation may not be necessary to the successful amelioration of bipolar symptoms, the question remains as to whether this effect, alone, is sufficient for bipolar therapy. Since other drugs are known to be effective in treatment of bipolar disorder without affecting MARCKS expression, and since non-therapeutic differentiating agents such as phorbols and retinoic acid are capable of eliciting MARCKS alterations, one may conclude that MARCKS down-regulation alone is not likely to lead to successful bipolar treatment in all cases. Indeed, lithium and VPA elicit other effects aside from MARCKS alterations, and such effects may also contribute to the overall mood-stabilizing action of these drugs.

As for the VPA-induced alterations in HN33 cell morphology and GAP-43 expression, such effects are not likely to be necessary or sufficient for the successful amelioration of bipolar symptoms. This is evidenced by the fact that lithium, the prototypic mood-stabilizing drug, had no effect on either parameter in our *in vitro* cell model, and that many agents may elicit GAP-43 alterations without necessarily exhibiting mood-stabilizing efficacy. One must question, then, the significance of the observed effects of VPA on these parameters, which seem to be related, based on previous findings. Numerous reports have emphasized GAP-43 as a protein integral to normal neuronal differentiation. Although the sequence of events is unknown, it is generally believed that cell differentiation is accompanied by a reduction in cell growth rates, the outgrowth of neuritic processes, and an increase in GAP-43 in neurons. The findings discussed herein

implicate VPA in invoking cellular differentiation, for all three of these parameters were observed. Perhaps VPA, by altering GAP-43 expression and cell growth/differentiative state, in turn alters the plasticity and function of areas in the brain that are integral to mood. According to such a scenario, the observed alterations in both MARCKS and GAP-43 may go hand-in-hand to elicit the plastic changes underlying clinical antimanic or mood-stabilizing efficacy. Indeed, animal models of epileptic kindling have shown a progressive and permanent alteration in neuronal structure and function. Perhaps a similar change underlies the pathology involved in bipolar disorder, so that for both diseases, VPA helps to bring about plasticity changes important in stabilizing the course of illness over time.

These findings are also suggestive of a mechanism to explain the developmental effects of VPA. It has been well established that prenatal VPA exposure results in a high frequency of neural tube defects, although the mechanistic basis for such effects is unclear. These data suggest that VPA elicits a change in the developmental program whereby cell growth is prematurely halted, prior to growth completion, and specialized functional differentiation begins before the tissue or organ has adequately prepared macroscopically for advanced developmental stages. As a result of such dramatic effects on functions (cell proliferation, differentiation, and substrate adhesivity) known to be integral to neural tube formation, the whole organism exhibits such gross abnormalities as open neural tube. Other reports are consistent with this hypothesis and the findings discussed herein (Wilk *et al.*, 1980; Mummery *et al.*, 1984; Martin and Regan, 1988; Martin *et al.*, 1988). In fact, the VPA-induced down-regulation of MARCKS also fits well and may contribute further to this model. As mentioned previously, mice lacking MARCKS due to antisense-mediated gene knockout die perinatally, exhibiting a number of gross abnormalities in midline structure formation (Stumpo *et al.*, 1995; Blackshear *et al.*, 1996).

Perhaps the ratio of MARCKS to GAP-43 is an important signal which, when out of balance, leads to a pathological, even fatal, phenotype. According to this hypothesis,

relatively higher MARCKS expression, concomitant with low GAP-43 expression, would signal for growth (cell division), and low MARCKS / high GAP-43 would signal for growth arrest and induction of differentiation. The data are thus far consistent with such a model, and future studies might further address the issue by examining and comparing relative MARCKS and GAP-43 levels in both control (unaffected) animals and those exhibiting abnormalities of the neural tube and other CNS regions. Such studies would be of great benefit medically, for they would help identify a mechanism for the development of neural tube defects in human offspring, and may even lead to diagnostic means and preventive measures to deal with such effects.

Expression of both GAP-43 and MARCKS is directly regulated by the enzyme PKC. If we understand better the role of PKC, we may also understand better the relevance of the observed VPA-induced effects. These studies have shown that PKC is clearly involved in the intracellular effects exhibited by VPA. However, PKC is apparently not central to the entire panorama of effects observed, for exposure of HN33 cells to inhibitors of PKC failed to halt the observed VPA-induced alterations in GAP-43 expression, and prevented the VPA-induced down-regulation of MARCKS only in the membrane, not cytosolic, fraction of cells. This raises the issue of how such effects could occur, especially in the case of GAP-43, if PKC is the primary regulator of both proteins. Previous studies have indicated that phosphorylation of GAP-43 is spatially and temporally restricted *in vivo* (Meiri *et al.*, 1991), and is implicated in LTP, a model for memory and learning (Stabel and Parker, 1991; Benowitz and Routtenberg, 1997; Son *et al.*, 1997), in part due to its association with state of growth and sprouting or neurite extension (Benowitz and Routtenberg, 1997). Meiri *et al.* (1998) showed that neurite outgrowth stimulated by neural cell adhesion molecules required GAP-43 phosphorylation. Yet another group showed that modulation of membrane-associated PKC activity played a necessary role in cell differentiation (Chakravarthy *et al.*, 1995). Such findings would suggest that PKC activity (specifically, phosphorylation of GAP-43)

is integral to neurite outgrowth. However, the role of PKC in neuronal differentiation remains in question. While there is substantial evidence for a positive role of PKC in regulating neuronal differentiation and maintaining the functionally active state (Coleman and Wooten, 1994; Cabell and Audesirk, 1993; Parrow *et al.*, 1992; Abraham *et al.*, 1991; Tonini *et al.*, 1991), and previous data have implicated a number of different PKC isoforms in cellular differentiation (Wada *et al.*, 1989; Leli *et al.*, 1992, 1993; Ways *et al.*, 1994; Hundle *et al.*, 1995; Borgatti *et al.*, 1996), other studies have suggested that, rather that being a positive regulator of the process, PKC may actually be an inhibitory influence, so that suppression of the enzyme results in increased process outgrowth (Heikkila *et al.*, 1989; Parodi *et al.*, 1990; Tsuneishi, 1992; Wooten, 1992; Carlson *et al.*, 1993; Jalava *et al.*, 1993; Ekinci and Shea, 1997). The results of the present experiments further argue against the notion that PKC is integral to cell differentiation, for addition of a PKC inhibitor to VPA-exposed HN33 cell cultures enhanced the neurite outgrowth and cell growth inhibition observed, and failed to reverse similar VPA-induced alterations in PC12 cells. Perhaps the cell differentiation parameters observed in this study, those of increased GAP-43 expression, decreased cell growth, and increased neurite outgrowth, occur concomitantly, and somehow signal to one another when the time is right to begin. In the case of the HN33 cells, VPA exposure may be leading merely to a slow in growth through altered expression of various cell cycling proteins, which in turn induces accompanying changes in cell morphology and GAP-43 expression. Or perhaps increased GAP-43 leads to neurite outgrowth, which then results in slowed division rate. This, again, is consistent with findings from studies of lithium, which had no effect on cell growth rates, and consequently had no effects on either cell morphology or GAP-43 expression. On the other hand, these VPA-induced effects on GAP-43 may be important for the kinds of plastic changes required for optimal anticonvulsant or mood-stabilizing therapy. The differences observed between lithium and VPA may explain their differing

abilities to treat various types of bipolar disorders, or the ability of VPA to effect partial amelioration of symptoms more quickly than lithium.

In PC12 cells, we investigated the potential of VPA to serve as a neuroprotective agent, since VPA exposure leads to cell differentiation, and has clearly been shown to be an efficacious drug for the treatment of either epilepsy or bipolar disorder. These two disorders are characterized by dysfunction of thought or electrical impulse, and one could hypothesize that VPA treatment brings about plastic changes important for either antiepileptic or mood-stabilizing therapy. In addition, lithium has been shown to have neuroprotective effects in various *in vitro* models. In this study, VPA exerted a protective influence against neurite retraction following trophic factor deprivation, while it had no protective effect on loss of cell number. Therefore, these data, while consistent with those discussed earlier (VPA decreased cell growth and increased neurite outgrowth), are inconclusive as to the potential for neuroprotection by VPA. Future studies may utilize other protection paradigms to address this issue.

In an effort to further the findings presented herein, one could investigate more in-depth the effects of VPA on PKC. It is yet unknown precisely how VPA interacts with this enzyme, or whether it is direct or indirect. As discussed previously, lithium affects PKC indirectly through its more direct inhibitory effects on PI signaling, which in turn lead to increased DAG-driven activation of PKC, as well as Ca^{++} release and further potentiation of PKC activation. VPA, on the other hand, is structurally very different from lithium, and it has been shown by these studies and others that VPA does not affect PI signaling. It would be useful to determine more definitively the mechanism by which VPA modulates PKC. Potential targets may be Ca^{++} release, which VPA has already been shown to affect, and which would in turn activate PKC; activation of an intermediate protein responsible for direct PKC activation; or simply via the enzyme itself, since VPA is a fatty acid, and some fatty acids are known to be activators of PKC (though one group has provided evidence against this hypothesis; Chen *et al.*, 1994).

Another important area of study related to differentiation is the investigation of the effects of VPA on gene regulation, for in addition to the role of PKC in altering protein expression by virtue of phosphorylation, PKC and other protein kinases (such as protein kinase A and tyrosine kinase), as well as protein phosphatases, are known to be involved in activation and inactivation of transcription factors AP-1 and CREB (Jackson, 1992; Karin and Smeal, 1992). AP-1 and CREB are known to modulate expression of key proteins which are involved in neuronal excitability, such as receptors, neuropeptides, and key enzymes in neurotransmitter biosynthesis, and these parameters are certainly of interest with regard to either epilepsy or bipolar disorder. Manji's group showed that in C6 glioma and human neuroblastoma (SH-SY5Y) cells, as well as in rat brain, VPA increased AP-1 DNA binding activity in both a time- and concentration-dependent manner, effects partially dependent upon PKC activity (Chen *et al.*, 1997). We have further shown evidence for VPA-induced alterations in MARCKS mRNA levels, also suggestive of effects on gene expression. This may be an important effect of VPA, or of mood-stabilizing agents in general. It may be that the efficacy of these anti-manic, mood-stabilizing drugs lies, in part, in their ability to influence and regulate expression of various genes. Retinoic acid, a fatty acid known to arrest growth and induce differentiation of a number of cell lines, is also known to affect gene expression through intracellular retinoic acid receptors which bind to gene promoter regions and modulate transcription (Watson *et al.*, 1994). It is of interest that retinoic acid has been found to elicit down-regulation of MARCKS expression in HN33 cells, which correlates well with findings reported herein for VPA: decreased MARCKS concomitant with decreased growth and increased differentiation. Furthermore, retinoic acid appears to elicit this effect without intermediary involvement of PKC (Watson *et al.*, 1994).

Yet another area of interest is the mechanistic basis for the developmental (*in utero*) effects of VPA, which, unfortunately, occur following therapeutic, not toxic, maternal plasma concentrations. In addition to the considerations mentioned above, the

metabolic nature of the VPA-induced effect should be examined more in depth. Previous studies suggest that while folic acid may prevent neural tube defects *in vivo*, this protective effect is not reproducible *in vitro*. Our preliminary studies in HN33 cells indicate that the protective effect of folic acid and its metabolites, such as methionine, are more complicated than merely inhibiting VPA-induced MARCKS down-regulation. Nonetheless, these findings are not inconsistent with the notion that VPA induces a significant alteration in the MARCKS/GAP-43 ratio which may underlie a pathological alteration in the developmental program of cells, leading to premature growth arrest and differentiation.

On the other hand, such modulation of cell growth may be considered therapeutic, rather than pathological, in other settings. For example, this anti-proliferative effect of VPA may prove useful in the area of cancer therapy. Other groups have already recognized this potential, and one report suggests an antitumor effect of VPA. In human neuroblastoma cells (UKF-NB-2 and UKF-NB-3), at nontoxic concentrations, VPA not only arrested cell growth and induced phenotypic differentiation, but also increased the immunogenicity of cells. This occurred through increased expression of surface neural cell adhesion molecules (NCAMs), and increased sensitivity to natural killer/lymphokine-activated killer (NK/LAK) cytolysis, through non-toxic mechanisms (Cinatl *et al.*, 1996). Therefore, VPA shows promise for treatment of cancer, and at concentrations already known to be well tolerated by patients.

With regard to experimental models, future studies might utilize primary cells derived from regions of brain thought to be important in affective disorders, such as the hippocampus. Even more importantly, *in vivo* animal experiments will be required before all of the effects reported thus far can be put into proper perspective with regard to their roles in the course and treatment of bipolar disorder. Animal studies, while quite instructive, are a considerably more difficult model in which to investigate the mechanism of action of VPA. Previous efforts in our laboratory have failed due to the difficulty

experienced in attempting to attain therapeutic serum levels of VPA, for the rat's metabolism is such that the drug is rapidly broken down and cleared following administration, with a half-life of about 1 h. This pharmacokinetic parameter is quite in contrast to the long half-life of VPA in humans, about 12 h.

VPA remains an invaluable drug for both the acute and prophylactic treatment of bipolar disorder, as well as for various seizure disorders. This drug has also proven quite useful in studying multiple aspects of the dysregulation involved in these diseases, and may one day lead to a clearer understanding of the pathophysiology which leads to bipolar disorder, as well as aid in the development of new and better drug therapies for the disorder.

Table 6.1. Effects of two mood-stabilizing drugs, VPA and lithium, as observed in HN33 cells following chronic exposure: A Summary. The presence of the described effect is indicated by "+", and "–" indicates the absence of the effect.

Effect	VPA	Lithium
↓MARCKS Protein	+	+ [a]
↓MARCKS mRNA	+	+ [b]
↓PI Signaling	–	+ [c]
↑GAP-43	+	– [b]
↓PKC Activity	+	+ [b]
↓PKC-α	+	+ [b]
↑PKC-δ	+	– [b]
↓PKC-ε	–	+ [b]
↑PKC-ζ	+	?
↓Cell Growth	+	– [d]
↑Neurite Outgrowth	+	– [d]

[a] Watson *et al.*, 1994
[b] Watson and Lenox, 1997
[c] Lenox *et al.*, 1996
[d] Watson and Lenox, 1996

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BIOGRAPHICAL SKETCH

The author was born in 1974 in Miami Shores, Florida, to two wonderful parents, John and Celeste Watterson. She was raised on the east coast of Florida, the oldest of three children. She earned her bachelor's of science degree in pre-medical biology at Miami's Barry University in 1995, graduating in three years with honors. She then began graduate studies in Pharmacology at the University of Florida, initially conducting research in the laboratory of Robert H. Lenox, M.D., and finishing under the guidance of Edwin M. Meyer, Ph.D. She is currently single and the proud "mommy" of a beautiful chocolate Labrador retriever-mix named Hershey.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edwin M. Meyer, Chair
Associate Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert H. Lenox, Cochair
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen P. Baker
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jeffrey K. Harrison
Associate Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Colin Sumners
Professor of Physiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1999

Dean, College of Medicine

Dean, Graduate School